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Chromosome Doubling Techniques For Production Of Doubled Haploids In *Brassica napus* L.

by

Albert John Hannig

A thesis submitted to the faculty of graduate studies and research in partial fulfillment of the requirements for the degree of Master of Science

in

Plant Breeding

Department of Plant Science

Edmonton, Alberta, Canada

Fall 1995

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Dr. Gary Stringam (Supervisor)

Dr. David Cass

Dr. Arnost Horak

Date: 29 August 1995

Abstract

The potential of antimicrotubule agents and cryo-treatments to induce chromosome doubling in microspores and microspore-derived embryos of *Brassica napus* L. cv. Topas was evaluated. Treatment of microspores with antimicrotubule agents resulted in increased embryogenesis and chromosome doubling frequencies relative to controls. Treatment of microspores with pronamide (15.0μM) and trifluralin (1.0μM), for 24 hours, produced doubling frequencies of 82.6 and 83.9% respectivly. Treatment of microspores with amiprophos-methyl, colchicine, and oryzalin, although effective, were less efficient because of increased toxicity associated with concentrations effective for chromosome doubling. Treating 30 day old embryos with oryzalin and trifluralin at a concentration of 10μM for four days, resulted in doubling frequencies of 78.6 and 61.1% respectively. However, treatment of embryos was determined to be less efficient for chromosome doubling than treatment of microspores because of the lengthy procedure for embryo treatment.

Cryo-treatment of freshly isolated microspores by cooling at a rate of -0.5^{0}C per minute to terminal temperatures of -15, -30, -45, and -60^{0}C, resulted in progressively increased chromosome doubling as the terminal temperature decreased. Cooling to a terminal temperature of -60^{0}C resulted in a maximum doubling frequency of 87.5%. Immersion of cooled samples in liquid nitrogen was lethal unless a terminal temperature of at least -45^{0}C was reached. Pre-treatment of microspores with ice (0^{0}C) for 24 hours before cryo-treatment significantly improved embryogenesis (survival) after liquid nitrogen storage. Ice pre-treated samples had increased doubling frequencies at higher terminal temperatures relative to microspores which were not pre-treated with ice.

Although cryo-treatment was shown to be less efficient than anti-microtubule herbicides for inducing chromosome doubling, the efficiency of germplasm storage was enhanced.

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List of Abbreviations

ABA-	abscisic acid.
AMA-	antimicrotubule agent.
AMH-	antimicrotubule herbicide.
APM-	amiprophos-methyl.
ATP-	adenosine triphosphate.
B5-	defined medium for washing microspores and *in vitro* culture of microspore-derived embryos (Appendix A,C).
DNA-	deoxyribonucleic acid.
GA-	gibberellic acid.
G1 phase-	gap 1 resting phase of the cell cycle.
G2 phase-	gap 2 resting phase of the cell cycle.
M phase-	mitotic phase of the cell cycle.
NLN-	defined culture medium for *in vitro* culture of microspores (Appendix B).
RNA-	ribonucleic acid.
S phase-	DNA synthesis phase of the cell cycle.

1.0 INTRODUCTION

Production of Doubled Haploids in *Brassica napus*

Production of homozygous plant lines is traditionally carried out by conventional breeding practices. This time-consuming process involves selection of parent lines with desirable agronomic traits followed by crossing, inbreeding and selection until superior inbred lines are developed. The traditional method is labor intensive and costly in resources and time commitments. Up to 10 years may be required before an inbred line is produced with conventional inbreeding.

When haploid plant chromosomes are doubled through colchicine treatment, much more rapid development of homozygous lines is possible. One can produce a homozygous line in a single generation without the time or cost associated with selection over a number of generations encountered with the traditional method. Haploid plants have been reported to occur spontaneously in field populations of *B. napus* at a rate varying from 0 to 6.85 haploid plants per 1000 (Thompson 1969; Stringam and Downey 1973). The frequency of haploids varies depending on the cultivar and is believed to be controlled by genetic elements. However, the low frequency of spontaneous haploid plants in field populations limits the usefulness of this source of haploids for plant breeding since the low numbers are insufficient to sample the genetic variability necessary for line development.

Although seed-derived haploids are of little use, other methods have been developed for producing large numbers of haploid plants. Isolated microspore culture, for example, is a highly effective means for producing haploid plants (Lichter 1982).

Microspore culture involves removal of microspores from floral tissues by selective filtration under sterile conditions. A small number of microspores, at a critical stage of development, have the capacity to change from gametophytic to sporophytic development. When placed in a defined culture media under proper inducing conditions, these microspores will develop into embryos. Since microspores have a gametic chromosome number, the majority of embryos which form are also haploid. The embryos eventually develop into mature plants which can be treated with colchicine to induce chromosome doubling.

In *B. napus*, isolated microspore culture was developed as a more efficient alternative to anther culture by Lichter (1982). Subsequently, numerous improvements have been made in the growth conditions of donor plants (Takahata et al. 1991; Lo and Pauls 1992), stage selection of microspores (Kott et al. 1987; Chuong et al. 1988; Telmer et al. 1992), culture conditions (Huang et al. 1990; Simmonds et al. 1991; Telmer et al. 1993; Custers et al. 1994) and regeneration of embryos into mature plants (Kott and Beversdorf 1990). These improvements have enabled microspore culture to become a practical method of producing haploid plants from a wide assortment of germplasm in *B. napus* and related genera (Siebel and Pauls 1989). Consequently, microspore culture is now utilized in many *B. napus* breeding programs.

Populations of microspore-derived plants may not consist entirely of haploids. In some genotypes as many as 80% of the plants may be diploid (Ahmad et al. 1991). On average, however, only 10 to 30% of microspore-derived plants are spontaneous diploids (Coventry et al. 1988; Chen and Beversdorf 1992b). Spontaneous diploids can be used

directly in breeding programs, assuming they have not arisen from unreduced gametes, as they do not require chromosome doubling or an extra generation of seed increase before selection begins. Chromosome numbers of haploid plants derived through isolated microspore culture have been doubled by treatment of whole plants with colchicine (Gland 1981; Coventry et al. 1988). The phytotoxicity of colchicine results in severe inhibition of plant growth and recovery. Approximately 60% of treated plants are able to produce at least one diploidized shoot (Gland 1981; Coventry et al. 1988). An extended period up to three months is usually required before harvest. Treated plants usually do not set seed prolifically, necessitating an extra generation of seed increase before enough seed is produced for field evaluation. Since colchicine is costly and may pose a significant health risk to individuals who handle it, further research into alternative chromosome doubling agents would be desirable.

Two alternatives to colchicine treatment which warrant investigation, include the application of antimicrotubule herbicides and cryo-treatments. Studies have demonstrated that these treatments can induce chromosome doubling in plant cells (Wan et al. 1991; Charne et al. 1988). Further research is required to determine if anti-microtubule herbicides and/or cryotreatments can be utilized in a practical protocol for chromosome doubling of haploid *B. napus*.

2.0 LITERATURE REVIEW

2.1 Target Site for Chromosome Doubling

2.1.1 Introduction

Treatments which are effective at inducing chromosome doubling in cells generally do so by interfering with microtubule function (Carter and Wick 1984; Morejohn and Fosket 1991). Properly functioning microtubules are required for a number of cellular processes including chromosome movement, cell plate formation and the completion of cell division. To develop better techniques for inducing chromosome doubling it is useful to have an understanding of the structure, dynamics and role of microtubules in plant cells.

2.1.2 Microtubule Structure

Microtubules are composed of tubulin heterodimers which polymerize to form microtubules. The heterodimer is composed of one alpha and one beta tubulin subunit which both have a molecular weight of approximately 55 kD (Appleby and Valverde 1989) and are composed of approximately 455 amino acids (Morejohn and Fosket 1991). Subunits are joined together in a head to tail fashion forming a linear protofilament which is 4-5 nm in width. Usually 13 protofilaments associate laterally in a 10 degree pitch to form a hollow microtubule which is 24 nm wide (Morejohn and Fosket 1991). Plant tubulin amino acid sequences differ from animal tubulin by 13 to 21% (Silflow et al. 1987). Plant alpha tubulin is slightly more distinct in terms of its amino acid sequence and electrophoretic mobility. Plant tubulin is also uniquely different from animal tubulin

in its immunological and pharmacological characteristics (Morejohn 1991). Consequently, plant and animal tubulin and microtubules vary in their response to destabilizing environmental and chemical agents.

Alpha and beta subunit genes exist as small multigene families. Isotypes of tubulin are produced from slight differences between the individual genes (Silflow et al. 1987). Post-translational modification of tubulin subunits involving acetylation or removal of carboxy terminal tyrosine residues (Hussey et al. 1987) can add further diversity to tubulin structure. Mutations causing single changes in the amino acid sequence of alpha tubulin in *Chlamydomonas* have resulted in variants with microtubules that are resistant to the effects of colchicine. Similarly, biotypes of goosegrass which are resistant to dinitroaniline herbicides have been inadvertantly selected through continued application of herbicides in cotton fields. These plants were also cross-resistant to phosphoric amide herbicides (Vaughn et al. 1987).

2.1.3 Microtubule Networks

During the course of mitosis there are four successive microtubule assemblies which form (Vaughn and Lehnen 1991). These include the interphase array of transverse microtubules, pre-prophase band, mitotic spindle and phragmoplast. The interphase array of transverse microtubules is located in the cortex of the cell and is arranged circumferentially in a helical fashion around the cell axis (Clayton 1985). Cellulose microfibrils in the cell wall often are oriented in a similar fashion. This array accomodates longitudinal cell growth (Marchant 1979). If treated with anti-microtubule

agents the interphase array and deposition pattern of cellulose microfibrils in the cell wall is disrupted (Morejohn and Fosket 1991). Consequently, cells become isodiametric in shape and meristematic regions may appear swollen and rounded (Vaughn and Lehnen 1991).

The pre-prophase band forms equatorially as a band of tightly packed microtubules in the cortex around the nucleus. This array of microtubules disperses at the start of prophase and anticipates the eventual cell division plane where a new cell wall will form (Cleary and Hardham 1988). In *B. napus* microspores undergoing pollen mitosis I, a pre-prophase band is not observable (Zaki and Dickinson 1990). The mitotic spindle apparatus allows for proper chromosome pairing to occur and separation into two equal haploid chromosome sets.

Cell division is completed by the phragmoplast microtubule array which forms the division plane between newly formed cells. Cellulose and other cell-forming material is deposited along the phragmoplast to form the new cell walls of the two cells. With the completion of cell wall formation during telophase, actively dividing cells enter into a new round of the cell cycle.

2.2 Chemical Antimicrotubule Agents

2.2.1 Introduction

Agents which inhibit the cell cycle usually fall into two classes: those which disrupt mitosis, or act at M phase, and those which prevent entry into mitosis or act at G1, S or G2 phases of the cell cycle (Hess 1987,1989). Agents which act at M usually

interfer with the formation and normal functioning of microtubules. Actively dividing cells are thus arrested in metaphase increasing the mitotic index. Upon recovery, these cells often bypass anaphase and telophase without dividing. In turn, this causes many cells to double in chromosome number as they complete another round of the cell cycle, a process known as endomitosis (Blackmore and Tootill 1984, Brodsky and Uryvaeva 1985). Examples of chemical agents which fall into this class include colchicine (Weerdenberg and Seagull 1988), dinitroaniline herbicides such as oryzalin (Cleary and Hardham 1988), and phosphoric amide herbicides such as amiprophos-methyl (Morejohn and Fosket 1984, Falconer and Seagull 1987) (Figure 2.1.1).

An absence of accumulated metaphases is usually observed with the second class of cell cycle disrupting agents. These agents tend to interfere with biochemical pathways. Metabolic reactions involved with amino acid, protein, ATP, RNA, and DNA synthesis, are most probable target sites. Because of the stage of the cell cycle at which these agents act, recovery from treatment usually does not result in chromosome doubling. However, with interference of DNA replication they may induce mutations as is seen with hydroxyurea (Barlow 1969). Examples of chemicals which fall into this class include protein synthesis inhibitors such as cyclohexamide (Moldave 1985), inhibitors of RNA synthesis such as actinomycin D (Webster and Van't Hof 1970) and inhibitors of DNA synthesis such as hydroxyurea (Barlow 1969).

2.2.2 Colchicine

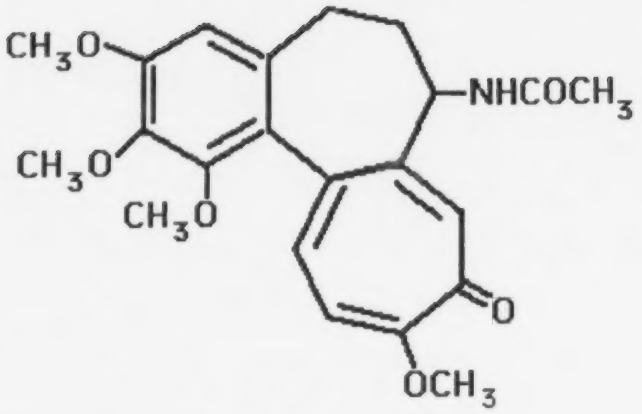
2.2.2.1 Description

Colchicine, (S)-N-(5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo[a]heptalen-7-yl)acetamide), is an alkaloid derived from the meadow saffron (*Colchicum autumnale*) (Hassawi and Liang 1991). Colchicine in low concentrations stimulates plant growth, whereas, higher concentrations, effective for chromosome doubling, retard growth (Walker 1938). This alkaloid reversibly binds to tubulin protein subunits and prevents their polymerization into microtubules (Andreu and Timasheff 1986; Engelsborghs and Fitzgerald 1986). Consequently, cellular processes which require microtubules are inhibited. Movement of organelles, chromosomes, and cell division in both plants and animals are all inhibited in cells treated with colchicine (Ilarslan and Aglan 1989).

2.2.2.2 Effective Concentration

A concentration of approximately 100 μM colchicine, applied for 6 to 8h, is required to induce metaphase arrest in endosperm tissue of African blood lily (*Haemanthus katherinae*) (Mole-Bajer 1958; Hepler and Jackson 1969). In onion (*Allium cepa*) a threshold concentration of 400 μM, applied for 4h, can induce c-mitosis in root meristem cells (Levan 1938). In tomato (*Lycopersicon esculentum*) concentrations of 500 to 1000 μM, applied for 6h, are required for metaphase arrest in root meristem cells (Barlow and Woodiwiss 1992).

a)



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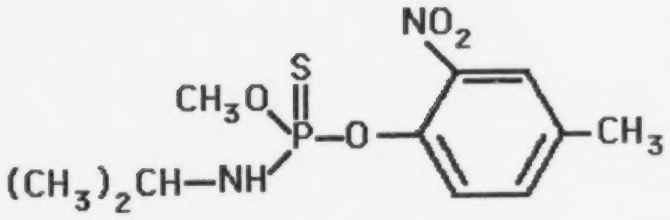

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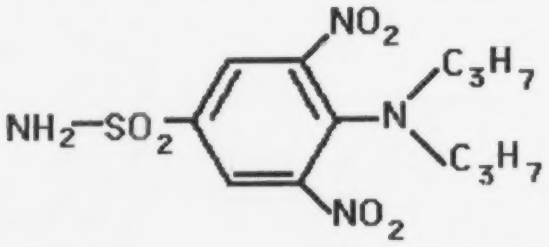

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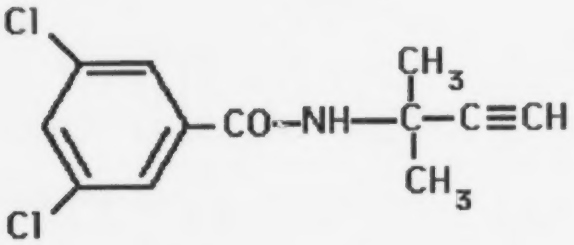

e)

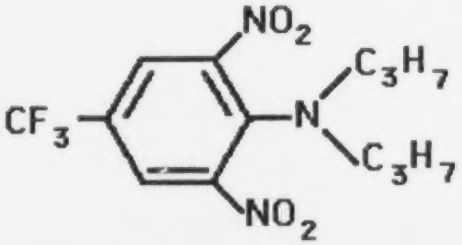

Figure 2.1.1 Molecular structures of anti-microtubule agents used to induce chromosome doubling in plant cells. a) colchicine, b) amiprophos-methyl, c) oryzalin, d) pronamide, e) trifluralin.

2.2.2.3 Application of Colchicine in Plant Breeding

Since 1937, when its properties as a chromosome doubling agent were discovered, colchicine has been the standard agent for chromosome doubling of plants (Blakeslee and Avery 1937). Colchicine was determined to be far more effective at inducing chromosome doubling than other agents investigated. Plant breeding programs have used colchicine primarily for the production of homozygous lines (doubled haploids) and for production of allotetraploids from hybrids. Different levels of polyploids, or multiples of the base chromosome number, can also be produced for plants which are propagated vegetatively, negating any problems that might occur during meiosis in plants that have higher ploidy level.

Protocols have been developed for canola (Gland 1981), gebera (Honkanen et al. 1992), maize (Wan et al. 1989), potato (Hermensen and Boer 1971), rice (Wong 1989), rye (Wenzel et al. 1977), soybean (Kollipara et al. 1989), sunflower (Gupta and Roy 1983), wheat (Barnabas et al. 1991; Taira et al. 1991) and a host of other crop plants. Colchicine solutions may consist of colchicine in water, or can be enhanced by altering pH and by the addition of dimethyl sulfoxide (DMSO) to aid in uptake (Taira et al. 1991). Usually, the roots of mature plants are placed into an aqueous solution of colchicine (0.025 to 0.5% w/v or 0.625 to 12.50 mM) for 3 to 6 hours. The aqueous colchicine moves to the shoot meristems with the transpiration stream. The roots are then washed and the plants potted in soil. Often the plants die back but recover after a few weeks of growth.

Colchicine treatment usually results in at least one or more doubled shoots per

plant in *B. napus*. More than 60% of treated plants usually produce doubled shoots (Coventry et al. 1988). Colchicine treatment often produces plants with chimaeral haploid and diploid tissue sectors. Although colchicine has been shown to cause chromosome anomalies and other types of mutation, the frequency of such events is thought to be too low to be of significance to breeding programs (Jensen 1974).

2.2.2.4 Application of Colchicine in *B. napus* Breeding

2.2.2.4.1 Treatment of Whole Plants

In *B. napus*, haploid plants derived from anther and microspore culture are usually doubled by treatment of mature plants with aqueous colchicine (Gland 1981; Coventry et al. 1988). This treatment has been employed for a number of years but is lacking in efficiency. Problems with treatment of mature plants include phytotoxicity, production of chimaeric plants, low doubling frequency, excessive cost and time required to produce homozygous doubled haploid lines. Treated plants often require three months to recover from treatment before flowering. Only 60% of plants may have a doubled shoot which in turn results in low seed set and the need for an extra generation of seed increase.

2.2.2.4.2 Treatment of Plantlets

Improvements over the treatment of mature plants have involved attempts to treat haploid material at earlier stages of development. Plantlets of *B. napus* (c.v. Duplo) treated at the 3 to 4 leaf stage with 50 mg/L (125 μM) of colchicine solution for 4 to 8 days have resulted in more than 50% of plants undergoing chromosome doubling

(Mathias and Röbbelen 1991). The best treatment involved treating plantlets with 50 mg/L (125 μM) of colchicine for 4 days which resulted in a survival frequency of 80% and a doubling frequency of 62.5%. Although doubling frequency with plantlets is not superior to whole plant treatment, treatment of plantlets improves the quality of doubled plants. These improvements include; complete chromosome doubling with absence of chimaeras, few problems with phytotoxicity, increased seed set, use of less colchicine and overall reduced costs for chromosome doubling.

2.2.2.4.3 Treatment of Microspores

Much better results are obtained when colchicine treatment is applied at the microspore stage of development. In the first series of studies on colchicine treatment of microspores the aim was not one of inducing chromosome doubling but to observe the effects colchicine had on cell division and embryogenesis (Zaki and Dickinson 1991). In this study, colchicine was used as an agent for disruption of microtubules of the microspore cytoskeleton and was aimed at determining if disruption of the first microspore division could affect microspore embryogenesis *B. napus*. Both highly embryogenic (c.v. Topas) and moderately embryogenic (c.v. Optima) cultivars were tested. Earlier studies on embryogenesis in *B. napus* (c.v. Topas) had shown that the first cell division of embryogenic microspores is symmetric rather than the normal asymmetric division which leads to formation of gametes (Zaki and Dickinson 1990). Application of colchicine was found to disrupt the microtubule network and cause a much higher proportion of microspores to undergo a symmetric division. Microspore cultures treated

with 0 to 25 mg/L (0 to 62.5 μM) colchicine, for 6, 12, 24 and continuously, produced a much higher number of embryos than did control cultures. The most effective treatment for embryo induction was the application of 25 mg/L (62.5 μM) colchicine for 12 hours (c.v. Topas) or for 24 hours (c.v. Optima). This resulted, on average, in more than 8,000 cotyledonary embryos per 200,000 treated microspores compared to less than 2,000 in the control for Topas and more than 25 in treated cultures compared with less than 5 in the control for Optima.

The effect of colchicine on embryogenesis in the *B. napus* cultivars Duplo, Janetzki, WP27 and F1 (Janetzki x Duplo) has also been tested (Iqbal et al. 1994). From 57 microspore culture experiments, 69% resulted in increased embryogenesis compared to the controls. In contrast to the results of Zaki and Dickinson (1991), the best overall treatment was found to be 100 mg/L (250 μM) for 24 hours. This treatment on average, produced three times as many embryos as in control cultures. No signs of toxicity on embryo development were observed in these experiments.

In another set of experiments, using the same genotypes as Iqbal et al. (1994), the effect of colchicine treatment on chromosome doubling was investigated (Möllers et al. 1994). Flow cytometric analysis indicated the best chromosome doubling treatment was application of 50 mg/L (125 μM) colchicine to microspore cultures for 24 hours resulting in 80 to 90% diploid embryos. Other researchers have also shown that direct treatment of isolated microspores with 0.05 to 0.1% (1.25 mM to 2.5 mM) colchicine for 15 to 22 hours can result in at least 70% chromosome doubling of regenerated plants (Chen et al. 1993). This treatment also caused increased embryogenesis, and has been utilized in line

development of canola quality *B. napus* since 1990.

The use of colchicine as an agent for chromosome doubling of microspore-derived *B. napus* has evolved to the point where it can be effectively applied at the microspore stage of development. A number of advantages occur when treating material at this stage. These include increased embryogenesis, little or no observable phytotoxicity, complete chromosome doubling, use of smaller amounts of colchicine, absence of chimaeric plants, no need for an extra generation of seed increase and an overall reduction in treatment costs. Obviously, treatment of microspores with colchicine will become a favored method for chromosome doubling of microspore-derived *B. napus* in breeding programs.

2.2.3 Antimicrotubule Herbicides

2.2.3.1 Amiprophos-methyl

2.2.3.1.1 Description

Amiprophos-methyl (APM), also known as BAY NTN 6867, is a phosphoric amide herbicide with the chemical name; O-methyl O-(4-methyl-2-nitrophenyl)(1-methylethyl) phosphoramidothioate. It was developed by Nitokuno (Tokyo, Japan) and Farbenfabriken Bayer GmbH (Leverkusen, Germany), and has been tested by Mobay Chemical Corporation (Kansas, U.S.A.). APM has been tested as a pre- emergence herbicide for control of grasses and broadleaf weeds and as a growth regulant to control formation of suckers in tobacco (Mobay 1980).

2.2.3.1.2 Effective Concentration

APM treatment of African blood lily (*Haemanthus katherinae*) endosperm resulted in rapid depolymerization of spindle microtubules (0.1 μM for <2 min.) (Bajer and Mole-Bajer 1986). In cell cultures of alfalfa (*Medicago sativa*) and zinnia (*Zinnia elegans*), a concentration of 1 to 3 μM induced complete depolymerization of both cortical and mitotic spindle microtubules (Falconer and Seagull 1987). APM has been effectively used for metaphase arrest and micro- nuclei induction in cell suspension lines of potato (*Solanum tuberosum*) (32 μM for 48h) and tobacco (*Nicotiana plumbaginifolia*) (32 μM for 24h) (Ramula et al. 1991; Verhoeven et al. 1990).

2.2.3.1.3 Application in Plant Breeding

APM has been tested for its ability to induce chromosome doubling in haploid maize callus (Wan et al. 1991). Concentrations varying from 1 to 20 μM were applied for three days. The best results were obtained with 15 μM applied for 3 days which resulted in a survival rate of 54.2% and doubling frequency of 67.0%. Unlike oryzalin and trifluralin, APM did not have an inhibitory effect on callus growth and development of plantlets. It was concluded that APM and pronamide were effective agents for producing doubled haploid plants in maize. To date, the use of amiprophos-methyl for chromosome doubling of microspore-derived *B. napus* has not been reported.

2.2.3.2 Oryzalin

2.2.3.2.1 Description

Oryzalin is a dinitroaniline herbicide with the chemical name; 4-(dipropylamino)-3,5-dinitrobenzenesulfonamide. It was developed by Eli Lilly and Company (Greenfield, U.S.A). Oryzalin is marketed under the trademark name Surflan as an aquaeous suspension or under the names Dirimal and Ryzelan as a dry concentrate. Oryzalin is used as a herbicide to control grasses and broadleaf weeds in crops such as soybean, cotton, potato, tobacco, grapes, turf and woody ornamentals. It is applied as a pre- or postemergent spray to the soil surface (Humburg 1989a).

2.2.3.2.2 Effective Concentration

Oryzalin causes rapid depolymerization of spindle microtubules in African blood lily (*Haemanthus katherinae*) endosperm at a concentration of (0.1 to 1.0 μM for < 2 minutes) (Bajer and Mole-Bajer 1986; Morejohn et al. 1987). A concentration of (1.0 μM for 6h) is required for complete metaphase arrest in the root meristem cells of tomato (*Lycopersicon esculentum*) (Barlow and Woodiwiss 1992). Treatment of roots resulted in complete depolymerization of microtubules in *Zinnia elegans* (10 μM for 40h) and *Lolium rigidum* (5 μM 1h) (Cleary and Hardham 1988). Oryzalin (30 μM applied for 30 h) was found to be highly effective for micronuclei induction in cell suspension cultures of potato (*Solanum tuberosum*) (Ramulu et al. 1991). Applied *in vitro*, oryzalin (7 μM) prevented taxol-induced polymerization of rose (*Rosa sp.*) tubulin into microtubules (Morejohn et al. 1987).

2.2.3.2.3 Application in Plant Breeding

Wan et al. (1991) have evaluated the application of oryzalin as a chromosome doubling agent for anther-derived haploid maize callus. Their best results were obtained with 5 μM oryzalin applied for 3 days. A survival rate of 52.2% (for transferred plantlets) and doubling frequency of 75.0% were obtained. In addition to the diploids, 8.3% tetraploid plants were produced. Oryzalin treatment was, however, considered to be somewhat toxic in that fewer plantlets developed from the original treated callus as compared to other anti-microtubule herbicides tested. It was concluded that oryzalin, along with trifluralin, may be too toxic to be an effective agent for the production of doubled haploid plants in maize.

Hassawi and Liang (1991) tested the chromosome doubling ability of oryzalin in wheat anther-derived haploid calli (cultivars "Kitt" and "Pavon"). Oryzalin treatment (10 μM for 72 hours) produced a doubling frequency of 50.0% in "Pavon". However, no doubled haploids were obtained in "Kitt". Oryzalin treatment was found to be inferior to that of colchicine. To date, the use of oryzalin as a chromosome doubling agent of microspore-derived *B. napus* has not been reported.

2.2.3.3 Pronamide

2.2.3.3.1 Description

Pronamide, a benzamide herbicide, has the chemical name; 3,5-dichloro-N-(1-dimethyl-2-propynyl)benzamide. Pronamide is also known as propyzamide and is marketed under the trademark name (Kerb 50 W) by Rohm and Haas (Philadelphia,

U.S.A.). Pronamide is used as a selective pre- and/or postemergence herbicide for control of grasses and broadleaf weeds in crops such as artichoke, blueberry, forage legumes, raspberry and woody ornamentals (Humburg 1989b).

2.2.3.3.2 Effective Concentration

Pronamide has been shown to induce metaphase arrest in *Allium cepa* roots at a concentration of (10 to 100 μM for 24h) (Vaughan and Vaughn 1987). Unlike dinitroaniline and phosphoric amide herbicides, pronamide treatment does not cause complete depolymerization of microtubules. Remnant microtubule "tufts" associated with the kinetochores of the chromosomes remain after treatment. The reason for the existence of these structures is still unknown.

2.2.3.3.3 Application in Plant Breeding

Wan et al. (1991) have evaluated the potential of pronamide as a chromosome doubling agent for treating anther-derived maize callus. Their best results were found when haploid callus tissue was treated with 5 μM pronamide for 3 days. This treatment resulted in a plant regeneration frequency of 50.5% and diplodization frequency of 73.5%. No higher levels of ploidy were observed with this treatment. This treatment did not inhibit callus growth and development of plantlets. It was concluded that pronamide and APM could be suitable for producing doubled haploid plants in maize. To date, the use of pronamide as a chromosome doubling agent of microspore-derived B. napus has not been reported.

2.2.3.4 Trifluralin

2.2.3.4.1 Description

Trifluralin is a dinitroaniline herbicide with the chemical name; 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)benzenamine. It was developed by Eli Lilly and Company (Greenfield, U.S.A.) and is marketed under the trademark names Treflan, Trefanocide and Elancolan. Trifluralin is used as a preemergent herbicide for the control of various grasses and broadleaf weeds in crops such as corn, mustard, sugarcane, wheat, barley, soybean, rapeseed, sugarbeets, tomato and other common field and greenhouse crops (Bayer et al. 1967; Humburg 1989c).

2.2.3.4.2 Effective Concentration

Treatment of liquid endosperm of African blood lily (*Haemanthus katherinae*) with trifluralin (0.3 μM for 15-30 min.) severly inhibited mitosis (Jackson and Stetler 1973). Trifluralin inhibited mitosis in the roots of *Allium cepa* (10 μM for 8 to 24 h) and *Triticum aestivum* (4.0 μM for 12 h) (Lignowski and Scott 1972). Trifluralin has been shown to bind to glass (Strachan and Hess 1982). Thus, observed results for effective concentration may be misleading.

2.2.3.4.3 Application in Plant Breeding

Wan et al. (1991) have evaluated trifluralin as a chromosome doubling agent for anther-derived haploid maize callus. Results were variable with application of 1 μM applied for 2 days, producing a survival rate of 42.2% for transferred plantlets and a

doubling frequency of only 19.8%. With 5 μM applied for 3 days, a survival rate of 50%, and doubling frequency of 100% were obtained. However, trifluralin proved to be toxic in that it inhibited the growth of regenerable callus and development of plantlets. It was concluded that trifluralin, when applied at its more effective higher concentrations, may be too toxic to effectively use as an agent for producing doubled haploid plants in maize.

Hassawi and Liang (1991) tested trifluralin on wheat anther-derived haploid calli (cv. Kitt and Pavon) for its ability to induce chromosome doubling. Trifluralin treatment, 10 μM for 72 hours, produced a doubling frequency of 57.14% in Pavon, however trifluralin treatment did not produce doubled haploids in Kitt. Trifluralin treatment was found to be inferior to colchicine treatment.

2.2.3.4.4 Application in *B. napus* Breeding

The use of trifluralin as a chromosome doubling agent of microspore-derived *B. napus* has been studied previously (Eikenberry 1993, 1994). Freshly isolated microspores were treated with trifluralin (0.5 to 1.0 μM for 24h) resulting in doubling frequencies in excess of 78% compared with 4.5 to 22.7% doubling in control cultures. Treatments also resulted in increased embryogenesis. Similar results were obtained with colchicine treatment of microspores, where embryogenesis was improved and doubling frequency exceeded 80% (Chen et al. 1993; Mollers et al. 1994). Because of its lower cost, ability to induce chromosome doubling in plant cells at lower concentrations, and reduced toxicity to humans, trifluralin can be considered as a more efficient alternative doubling agent than colchicine.

2.3 Cryo-treatment

2.3.1 Introduction

Cryopreservation is a term used to describe the storage of biological material at low temperatures, and usually refers to storing material in liquid nitrogen vapour (-140^{0}C) or immersed in liquid nitrogen (-196^{0}C)(Withers 1985). At these low temperatures, no biologically significant chemical changes are believed to occur and cells are in a state of suspended animation. Cryo-treatment, in the context of this thesis, is a term used to define low temperature treatments, including cryo-preservation, to which biological material is exposed. This term is used broadly to include the testing of parts, or the whole of a cryopreservation protocol used to determine which factors are responsible for an observed response.

2.3.2 Cryopreservation of B. napus Germplasm

B. napus germplasm has been cryopreserved by a number of methods. Shoot tips (Withers et al 1988), embryos (Uragami et al. 1993), cell suspensions (Weber et al. 1982), and microspores (Charne et al. 1988; Chen and Beversdorf 1992a,b) have all been cryopreserved. Most of these techniques involve preculturing of plant material in cryoprotectant media followed by direct immersion in liquid nitrogen (vitrification) or slow cooling to approximately -40^{0}C before immersion in liquid nitrogen. Survival rates in excess of 50% have been reported for most of the above techniques with some protocols resulting in excess of 90% survival. However, difficulties often exist in regeneration of plants. Problems with removal of cryoprotectants, and excessive callusing

of tissue can lead to inhibition of recovery and growth.

To date, the most successful techniques for storing *B. napus* germplasm do not employ cryoprotective agents, other than those agents found in the regular culture medium. The most effective *B. napus* germplasm storage techniques include either the use of air-dried microspore-derived embryos and/or slow cooling of microspores followed by cryostorage (Charne et al. 1988; Chen and Beversdorf 1992a,b). Cryopreservation of air-dried microspore-derived embryos encapsulated in calcium-alginate gel also produces high survival rates which exceed 90% (Uragami et al. 1993). However, since it is difficult to remove the gel, which hinders regeneration of embryos into plants, the efficiency of this technique requires further improvement.

2.3.3 Cold Acclimation in *B. napus*

Cells, embryos and whole plants of *B. napus* can be stimulated to undergo various changes associated with cold acclimation. These changes can lead to increased ability to withstand the damaging effects of a freeze-thaw cycle.

As with other cold acclimated plant material, acclimated *B. napus* cells have been shown to undergo adaptive changes to freezing stress. Alterations in the fatty acid composition of the plasma membrane occur (Diepenbrock 1984; Williams et al. 1992). Cold acclimation induces the synthesis of new proteins that are believed to be associated with freezing tolerance (Johnson-Flanagan and Singh 1987; Parra et al. 1990; Saez-Vasquez et al. 1993). Similarly, decreases in other proteins such as the rubisco small subunit also occur (Meza-Basso et al. 1986).

Treatment of *B. napus* cells, embryos and seeds with ABA has been shown to cause increased tolerance to freezing and dessication (Harada et al. 1989; Orr et al. (1986, 1990); Johnson-Flanagan et al. (1991, 1992)). ABA treatment resulted in the expression of new proteins associated with freezing tolerance (Weretilnyk et al. 1993).

2.3.4 Cryo-induced Chromosome Doubling in *B. napus*

Factors which interfere with the cytoskeleton of microspores can increase the number of microspores which undergo embryogenesis (Zaki and Dickinson 1991; Lo and Pauls 1992; Custers et al. 1994; Iqbal et al. 1994). The same factors can also increase the percentage of doubled haploid plants which are produced from microspore culture.

Cryopreservation of plant cells is generally considered to be an ideal way to store germplasm in a genetically stable form and is not normally associated with chromosome doubling (Withers 1984). Differences between cell types may exist which protect or predispose cells to chromosome doubling. Cryo-induced chromosome doubling has only been shown to occur when late uninucleate stage microspores are cryopreserved (Charne et al. 1988; Chen and Beversdorf 1992a). However, it has been known for several years that treating plants with temperature shocks can result in production of unreduced gametes (Sax 1936, 1937).

The mechanism by which cryopreservation induces chromosome doubling at the microspore stage of development is not yet known. Some researchers have suggested that chromosome doubling in *B. napus* microspores may result from endoreplication or endomitosis (Keller et al. 1975). Others have suggested that cryo-selection of pre-existing

unreduced microspores may be involved (Chen and Beversdorf 1992a). However, it is unlikely that larger cells, such as unreduced gametes, would have a selective advantage. The larger volume of these cells prevents removal of water which should lead to more ice-damage and increased mortality. Most likely, it is sub-lethal cryo-damage to components of the cytoskeleton, such as tubulin protein, that is involved. Cold-induced denaturation of microtubule subunits accompanied by irreversible precipitation has been shown to occur in onion and spinach (Carter and Wick 1984; Bartolo and Carter 1991a,b). Dehydration of the cytoplasm during cooling, which is dependent on terminal cooling temperature, can also cause precipitation and damage to proteins. Similarly, chromosome doubling frequency in *B. napus* has been shown to increase as the terminal cooling temperature decreases during the cryopreservation protocol (Chen and Beversdorf 1992a). As with anti-microtubule chemicals, it is possible that cryo-damage and inhibition of microtubule formation results in chromosome doubling of microspores during the first proembryo division. Such an event can be considered to be equivalent to endomitosis as induced by colchicine (Blackmore and Tootill 1984; Brodsky and Uryvaeva 1985). Further research is required to characterize the events which lead up to chromosome doubling in *B. napus* microspores.

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3.0 EXPERIMENTS

3.1 Experiment #1: Chromosome Doubling of Microspores and Production of Doubled Haploid Plants in *Brassica napus* L. cv. Topas by Antimicrotubule Agents.

3.1.1 Abstract

The potential use of four different antimicrotubule herbicides as agents for chromosome doubling in *B. napus* L. cv. Topas were investigated. Microspores were treated separately with amiprophos-methyl, oryzalin, pronamide and trifluralin at concentrations varying from 0.05 to 50 μM for a period of 24 hours. The chromosome doubling ability of these agents was compared to that of colchicine, a well known agent for chromosome doubling in plants. With amiprophos-methyl (10.0 μM), oryzalin (0.1 μM), pronamide (15.0 μM), trifluralin (1.0 μM), and colchicine (1.25 mM), treatments induced doubling frequencies of 75.0, 58.8, 82.6, 83.9 and 80.4% respectively. Pronamide and trifluralin treatments were superior to that of colchicine. Herbicide treatments increased embryo yields and exhibited varying degrees of toxicity, as determined by decreasing embryo yields, as concentrations increased. From all of the treatments only low numbers of polyploids and no chimaeric plants were produced. Because of reduced toxicity, cost, and chromosome doubling ability, antimicrotubule herbicides are considered an effective alternative to colchicine treatment for chromosome doubling in *B. napus*.

3.1.2 Introduction

Isolated microspore culture is a rapid and efficient method for producing haploid plants in *B. napus* breeding programs. Following chromosome doubling of haploid

microspore-derived plants, homozygous lines can be produced within a single generation. Populations of microspore-derived plants are composed of only 10 to 30% spontaneous diploid plants (Coventry et. al. 1988; Chen and Beversdorf 1992). Consequently, most microspore-derived plants require treatment to induce chromosome doubling. This process has traditionally involved treating plants at the early flowering stages with colchicine. Colchicine treatment induces at least one doubled shoot in approximately 60% or more of treated plants (Gland 1981). However, this method is lacking in efficiency. Plants often require three months recovery after treatment, sectorial chimeras are often produced, seed set is usually very low, and typically a further generation for seed increase is needed.

To overcome inefficiencies with colchicine treatment of whole plants, it is useful to investigate the potential of alternative chemical agents applied at various developmental stages for chromosome doubling. Recent experimental work has demonstrated that colchicine could be applied to freshly isolated microspores. Not only did this treatment increase the percentage of spontaneously doubled microspore-derived plants to more than 80%, but it also caused a larger percentage of cultured microspores to undergo sporophytic (embryogenic) development (Zaki and Dickinson 1991; Iqbal et al. 1994; Möllers et al. 1994). It has been demonstrated that colchicine mediates its effects by binding to tubulin subunits and inhibiting their polymerization into microtubules (Andreu and Timasheff 1986). In the absence of microtubules, chromosome movement during mitosis, cell plate formation, and cell division are inhibited. This is known as a colchicine or c-mitosis. Since the chromosome cycle is not affected, inhibition of microtubule polymerization during the cell cycle, often results in cells which have twice the normal

chromosome complement.

The antimicrotubule herbicides amiprophos-methyl (APM), oryzalin, pronamide and trifluralin, like colchicine, bind to tubulin subunits and inhibit the formation of microtubules in plant cells (Hess 1989, Vaughn and Lehnen 1991). However, these agents have a higher binding specificity for plant tubulin than does colchicine. While colchicine inhibits microtubule formation in plant cells at mM concentrations, antimicrotubule herbicides are effective at μM concentrations (Morejohn and Fosket 1991; Vaughn and Lehnen 1991). Consequently, there is potential for antimicrotubule herbicides to become effective alternatives to colchicine for chromosome doubling in *B. napus*. The main objective of this experiment was to determine the ability of APM, oryzalin, pronamide, trifluralin and colchicine to induce diploidization of *B. napus* L. cv. Topas at the microspore stage of development.

3.1.3 Materials and Methods

Growth of Microspore Donor Plants

Procedures followed for growing donor plants and microspore culture were those of (Coventry et al. 1988) and (Swanson 1990). Seeds of *B. napus* L. cv. Topas were germinated at 18^{0}C on moist filter paper in petri dishes. Seedlings were transferred to 6-inch pots containing metro mix (W.R. Grace & Co., Canada) soil-free growth media. The pots were placed in a growth cabinet maintained at 24/21^{0}C day/night temperature with a 16 hour photoperiod and photon flux density of 425-450 μmol m^{-2} s^{-1}. After reaching the four-leaf stage, the number of plants per pot were thinned to one. Plants

were watered as required, 3 to 4 times weekly, with a solution containing 1 g/L of(20-20-20 [N:P:K]) fertilizer. As the first buds began to form, the temperature in the growth chamber was reduced to 10/5^{0}C day/night.

Microspore Isolation

Racemes with buds at the proper stage of development (1.5-3.5mm in length) were excised from donor plants, wrapped in moist paper towel, covered loosely with chopped ice and placed in a fridge in the dark at 0-1^{0}C for 24 hours. The buds were then surface sterilized in a solution of sodium hypochlorite 7.0% (w/v) for 10 minutes and rinsed three times for 5 minutes with sterile ice cold water. The buds were gently crushed with a pestle and mortar containing B5 isolation medium (Appendix A) (Lichter 1982) modified with 13% (w/v) sucrose, and the homogenate filtered through 63 and 44 µm nylon mesh (Nytex). The filtrate was then poured into a 50 mL Falcon tube (Fisher Scientific, Canada) and centrifuged at 250g for 10 minutes. The microspore pellet was resuspended and centrifuged twice with B5 medium and resuspended in modified NLN medium (Appendix B) (Lichter 1982), containing 13% sucrose, (@ 1 mL per isolated bud).

Treatment of Microspores With Anti-microtubule Agents

Amiprophos-methyl was provided by the Mobay Chemical Co. (Kansas City, U.S.A.), pronamide by Rohm and Haas Co. (Philadelphia, U.S.A.), oryzalin and trifluralin by Dow Chemical Co. (Midland, U.S.A.). Aliquots of antimicrotubule herbicides, from 4 mM stock solutions dissolved in acetone, were added to separate microspore samples

in Falcon tubes. Final concentrations of 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 15.0, 30.0 and 50.0 μM were obtained. Colchicine obtained from a 1mg/mL filter-sterilized stock solution dissolved in NLN medium, was added to separate samples to produce a final concentration of 1.25 mM (0.05% w/v) colchicine. The concentrations of the antimicrotubule agents tested were within the range known to induce microtubule depolymerization in plants (Morejohn and Fosket 1991). Falcon tubes containing the microspores with herbicides and colchicine were placed in an incubator at 30°C in the dark.

Post-treatment Culture

After 24 hours, samples were washed with NLN medium and centrifuged 3 times. The microspore concentration was adjusted, with a haemocytometer, to approximately 6x 10^4 microspores/mL NLN and 10 mL aliquots were placed into (100x 15mm) petri plates. The plates were sealed with parafilm and placed in an incubator in the dark at 30°C. After 14 days the plates were placed on a rotary shaker (60 rpm) at room temperature in the dark. At 30 days of development the numbers of cotyledonary embryos were recorded and the embryos transferred to petri plates (100x 15mm) containing B5 solid media supplemented with 0.15 mg/L gibberellic acid (Appendix C). Each plate, containing 20 embryos, was first incubated for 10 days at 4°C with an 8 hour photoperiod and photon flux density of 30 μmol m^{-2} s^{-1}, followed by incubation at room temperature with a 12 hour photoperiod. The plates were then transferred to room temperature (24-25°C), with a photoperiod of 12 hours and photon flux density of 30 μmol m^{-2} s^{-1}, until the embryos

germinated and formed true leaves (30-40 days). The numbers of surviving plants per treatment were recorded.

Plantlet Transfer and Plant Regeneration

Plantlets (germinated embryos) were transferred to multiwell transfer flats containing moist soil-free mix (metro mix). Plantlets were watered, misted and covered with clear plastic to maintain humidity. After one week the plastic was removed and the plantlets irrigated regularly with a solution containing 1 g/L of (20-20-20 [N:P:K]) fertilizer.

Ploidy Determination

Ploidy levels were determined by counting the chloroplast number in guard cells of expanded leaves (Chevre et al. 1989; Lucas et al. 1991). Two leaf disks per plant were removed from the abaxial surface and placed on a glass slide with the abaxial side facing upwards. A few drops of 1.0% (w/v) silver nitrate solution were placed on each disk for 3 to 5 minutes. A glass coverslip was then placed over the disks and guard cell chloroplasts, in stomates around the outer edge of the leaf disks, were counted under the magnification of a standard light microscope.

To verify the guard cell chloroplast method of ploidy determination, meiotic chromosome numbers of ten each of haploid, diploid and polyploid plants were observed. Chromosomes were stained as recommended by Viinikka and Sovero (1988). Immature flower buds were fixed in 6:3:1 (ethanol: acetic acid: chloroform) for 24 hours or longer

followed by transfer into ethanol solutions of 70% (12 hours), 50% (30 minutes) and 30% (5 minutes). Buds were placed in water (5 minutes), hydrolyzed in 1N HCl at 60^{0}C (8 minutes), rinsed with water and stained with dilute Schiffs reagent for 45 minutes. The buds were rinsed with water and dissected in 45% acetic acid, and a cover slip placed over the dissected material and gently pressed. Slides were breifly placed in liquid nitrogen and the cover slip removed. Slides were placed in absolute ethanol (5 minutes), acetic acid (25 seconds) and allowed to dry. Slides were rinsed with Sorenson's phosphate buffer (pH 6.8) (5 minutes), stained with 4% Giemsa stain in the same buffer solution (15 minutes), rinsed with water and allowed to dry. Specimens were mounted in permount followed by observation under a light microscope.

Statistical Analysis

The experiment was repeated twice, each with two replicates of 20 transferred embryos per treatment; a total of 80 embryos per treatment were transferred. The means of the various treatments were compared to the effectiveness of colchicine as an agent for chromosome doubling using Duncan's new multiple range test (Steel and Torrie 1980).

3.1.4 Results

Plant Population Regenerated

Seven hundred and forty-six plants were produced from all treatments. Of these, 235 (31.5%) were haploid, 495 (66.4%) diploid and 16 (2.1%) were tetraploid. No mixoploids were observed in the plants regenerated.

Effect of Anti-microtubule Agents on Embryogenesis

From the comparison of means (Duncan's multiple range test) values for embryo yield which are equal to or higher than 142.9 % can be considered to be significantly different, or improved, relative to the control value of 100.0% (Table 3.1.1).

Control cultures produced an average of 742 embryos (per 6x 10^5 plated microspores) (Table 3.1.1). Acetone was not added to the controls as it had been previously demonstrated that, at the concentrations used to dissolve herbicides, it has little effect on embryogenesis (Eikenberry 1994). Microspores treated with 1.25 mM (0.05% w/v) colchicine produced an average of 620 embryos or 83.6% of the number obtained with the control (Figure 3.1.1).

Amiprophos-methyl treatments resulted in increased embryogenesis at concentrations of 0.1 µM (902 embryos or 121.6% of controls) and 0.5 µM (844 embryos or 113.7% of controls) (Figure 3.1.2). Concentrations of 1.0 µM and higher resulted in progressively decreased embryogenesis (increased toxicity) compared to the control. At 15.0 µM no embryos were produced. Application of oryzalin at a concentration of 0.1 µM resulted in an average of 1154 embryos (155.5%) (Figure 3.1.3). At 0.5 µM embryogenesis was decreased to 676 embryos (91.1%). Concentrations of 1.0 µM and higher resulted in very few or no embryos. Pronamide treatments resulted in very positive effects on embryogenesis (Figure 3.1.4). The lowest concentration of 0.1 µM resulted in an average of 1060 embryos (142.9%), whereas the highest concentration of 50 µM resulted in an average of 1346 embryos (181.4%). This later value was the highest obtained of all of the treatments. Unlike the other agents tested, pronamide treatments did

not appear to induce toxicity effects as determined by embryogenic frequency. Trifluralin treatments produced embryogenic frequencies superior to that of controls up to a concentration of 1.0 μM (Figure 3.1.5). Highest embryogenic frequencies were obtained at 0.05 μM (1204 embryos (162.3%)) and 0.1 μM (1210 embryos (163.1%)). Embryogenesis began to decrease at a concentration of 5.0 μM (474 embryos (63.9%)). At concentrations of 10.0 μM and higher very few or no embryos were produced.

Overall, pronamide treatments had the most positive effect on embryogenesis. At the concentrations tested pronamide did not produce symptoms of toxicity. Amiprophos-methyl and trifluralin appeared to be similar in terms of toxicity. Trifluralin had a positive effect on embryogenesis only at low concentrations (0.05 and 0.1 μM). Oryzalin treatments had the least positive effect on embryogenesis. At 0.1 μM embryogenesis was improved relative to the control but at 0.5 μM, and especially at higher concentrations, embryogenic frequencies rapidly decreased.

Effect of Anti-microtubule Agents on Plant Regeneration

A comparison of means (Duncan's multiple range test) indicated that most of the treatments were not significantly different from plant regeneration in the control (Table 3.1.1). In this experiment plant regeneration values of 20.0% or less were considered to be significantly different (@ P= 0.05), or inhibitory, relative to the control.

Control treatments resulted in an average of 53.8% plant regeneration, whereas colchicine (1.25 mM) treatments produced an average regeneration rate of 47.5% (Figure 3.1.1). APM treatments produced the lowest regeneration frequencies at a concentration

of 10.0 μM (16.3%) and highest regeneration at 1.0 μM (53.8%) (Figure 3.1.2). Oryzalin produced its lowest regeneration frequencies at 0.1 μM (15.0%) and highest at 0.5 μM (28.8%) (Figure 3.1.3). Higher concentrations of oryzalin inhibited embryogenesis such that further regeneration of plants was not carried out. With pronamide, the lowest regeneration frequencies were obtained at a concentration of 0.1 μM (20.0%) and the highest at 50.0 μM (78.8%) (Figure 3.1.4). For trifluralin 0.5 and 5.0 μM both produced the lowest regeneration frequency (27.5%), while the highest regeneration was obtained at a concentration of 0.05 μM (67.5%) (Figure 3.1.5). Plant regeneration varied considerably for the various treatments. There was no consistent relationship between herbicide treatment, concentration and plant regeneration. Similarly, embryo number did not appear to influence the ability of embryos to germinate and develop into plants. It might be expected that in cultures with lower embryo number, the greater availability of nutrients would result in embryos of better quality. Although embryos in such cultures were larger they did not appear to have any increased ability to germinate when compared to embryos developing from more embryogenic cultures.

Although the anti-microtubule agents were removed from cultures only after 24 hours of treatment, they did not appear to have any lasting effect on the morphology or development of embryos derived from surviving microspores. Overall, plant regeneration was not considered to be adversely affected by the various treatments. Embryos were not subjected to any treatments which could have increased germination and regeneration frequencies other than that recommended in the standard protocol. Dessication and/or altering low temperature treatment (0 to 4^0C) of embryos have been reported to

significantly improve germination frequencies to more than 90.0% (Kott and Beversdorf 1990). In a breeding program, such treatments should be used to improve germination and plant regeneration frequencies.

Effect of Anti-microtubule Herbicides on Diploidization

Control treatments produced an average of 35.5% diploid plants with the remainder being haploid (Table 3.1.1). No tetraploids were produced from control cultures. Colchicine treatment at 1.25 mM resulted in an average diploidization frequency of 80.4% (Figure 3.1.1). APM treatment resulted in the lowest diploidization at 0.1 μM (43.4%) and highest at 10.0 μM (75.0%) (Figure 3.1.2). Diploidization frequency tended to increase as APM concentration increased. Oryzalin produced diploidization rates of 58.8% (@ 0.1 μM) and 46.4% (@ 0.5 μM) (Figure 3.1.3). Pronamide treatments tended to have increased diploidization as concentration increased and ranged from a low at 0.5 μM (48.0%) to a high at 15.0 μM (82.6%) (Figure 3.1.4). Similarly, with trifluralin, increased concentration tended to increase the frequency of diploidization (Figure 3.1.5). Diploidization ranged from a low at 0.05 μM (50.8%) to a high at 1.0 μM (83.9%).

From the comparison of means (Duncan's multiple range test) values for diploidization which are equal to or higher than 65.5% are significantly different, or improved, relative to the control value of 35.5% (Table 3.3.1). These treatments are therefore considered to be effective for improving diploidization frequencies.

Autotetraploids

In general, tetraploid plants occurred at very low frequencies. At the concentrations used and period of duration (24 h), a total of 16 autotetraploid plants were produced for all treatments. The numbers for given treatment agent were as follows; control (0), colchicine (1), APM (2), oryzalin (0), pronamide (4) and trifluralin (9). Although trifluralin at 5.0 μM produced 4 tetraploid plants, or 18.2% of the total plants regenerated from the 5.0 μM treatment, for most of the treatments no clear relationship appeared to exist between increased concentration of herbicide and increased production of tetraploid plants. Tetraploid plants can be easily recognized by morphological features and tend to set seed poorly. Tetraploid plant production does not appear to be a significant factor in application of anti-microtubule agents for chromosome doubling. Although, in theory, the frequency of tetraploids should increase as herbicide concentration and treatment duration increase, herbicide toxicity tends to increase as well. In turn, this tends to eliminate the use of treatments involving higher concentrations of herbicide. Ploidy levels higher than the tetraploid level were never observed.

Seed Set

Most plants were not grown to full maturity since ploidy levels were determined during vegetative phases of development. Diploid plants set seed in a fashion similar to normal seed-derived plants. Unlike plants which had been doubled at early bud stages, microspore doubling produced plants which were of only one ploidy level. Chimaeric (mixoploid) plants, which do not set seed well, were never observed in any of the

treatments. Haploid plants produced vestigial buds which atrophied with no pod or seed formation occurring. Tetraploid plants produced pollen abundantly but had very low seed set. Pods which formed often did not fill with seed or had deformed seeds. Some pods formed one or two large seeds which could be germinated.

Plant Morphology

The ploidy level of the microspore-derived plants directly affected the morphology of mature plants. The primary means of ploidy level determination was by staining leaf disks with silver nitrate to determine the number of chloroplasts per guard cell set (Chevre et al. 1989; Lucas et al. 1991). Haploid plants had 8 chloroplasts per guard cell set, whereas diploids and tetraploids have 16 and 32 chloroplasts respectively (Figure 3.1.8). In most cases the chloroplasts were divided equally between the two guard cells, but some variation occurred. Guard cell size also increased as the ploidy level increased.

The ploidy level of *B. napus* plants had a direct effect on vegetative characteristics. Haploid plants were smaller, had stems and leaves that were thin and formed small buds in comparison to diploids (Figure 3.1.6). Although haploid plants produced flowers, they were sterile and did not produce pollen (Figure 3.1.7). Diploid plants had larger and thicker stems, leaves, and flower buds (Figure 3.1.6). The flowers were larger than those of haploids (Figure 3.1.7), diploid anthers produced pollen, and pod formation and seed set occurred with few problems. Tetraploid plants did not appear to be larger than diploids, but they had thicker stems, leaves, and flower buds (Figure 3.1.6). Tetraploids tended to have fewer leaves which were leathery, less elongated and had veins

which were wider and thicker than those of diploid leaves. The flowers of these plants were similar in size to those of diploids but the petals were less elongate, appeared to be ruffled, and had stronger pigmentation (Figure 3.1.7).

Ploidy determinations by guard cell chloroplast counts were verified by correlation with chromosome numbers (Figure 3.1.9). Chromosome numbers from haploid, diploid and tetraploid plants were (2n=2x=19), (2n=4x=38) and (2n=8x=76) respectively.

3.1.5 Discussion

The present study illustrates the usefulness and varying efficiencies anti-microtubule agents have for inducing chromosome doubling in *B. napus* at the microspore stage of development. As with colchicine, antimicrotubule herbicides such as APM, oryzalin, pronamide and trifluralin bind to tubulin subunits which are normally polymerized into microtubules. Consequently, microtubule formation and the cellular processes they mediate are inhibited. Treatment of actively dividing plant cells with antimicrotubule agents induces events synonymous with c-mitosis (colchicine mitosis) (Levan 1938). Microtubule-mediated processes in these cells, such as formation of the mitotic spindle, chromosome movement, and cell plate formation, cease to occur resulting in the formation of restitution nuclei and inhibition of cell division (Morejohn and Fosket 1991). Since the chromosome cycle is unaffected DNA replication occurs normally during the subsequent S-phase of the cell cycle. Consequently, a cell with twice the normal chromosome number is formed. This process can be considered to be a type of chemically-induced endomitosis (Brodsky and Uryvaeva 1985). Treatment of cells with

antimicrotubule chemicals for periods exceeding one round of the cell cycle may result in several endo-cycles in which cells of increasingly higher levels of ploidy may be formed.

To increase the efficiency of chromosome doubling in *B. napus*, treatment of embryogenic microspores with antimicrotubule agents during the first embryogenic division may result in a simple and rapid method of doubling. Antimicrotubule herbicides such as APM, oryzalin, pronamide and trifluralin are effective at inhibiting cell division in plants at μM concentrations compared to colchicine, which is effective at mM concentrations (Morejohn and Fosket 1986,1991; Hess 1987). In addition to their specificity for plant cells, antimicrotubule herbicides do not bind to animal tubulin and therefore are much less toxic to humans. Colchicine, on the other hand, binds to animal tubulin at a 1000x lower concentration than is required for plants, it therefore can pose a considerable health hazard if not handled properly (Hart and Sabnis 1976; Morejohn and Fosket 1986).

To solve problems associated with whole plant treatment, initial experimental work has focused on applying colchicine at earlier stages of development. Mathias and Röbbelen (1991) found that immersing the roots of plantlets (3-4 leaf stage) in 50 mg/l (125 μM) colchicine for 4 days resulted in a survival rate of 80.0% and a doubling frequency of 62.5%. Although this did not produce increased chromosome doubling over whole plant treatment, treated plantlets developed into completely doubled plants (as determined by flower morphology), did not suffer toxicity effects, and achieved a relatively high rate of seed set.

Application of colchicine treatment at the microspore stage of development was first investigated by Zaki and Dickinson (1991). Their study was initially designed to observe the effect colchicine had on the first embryogenic division of microspores and subsequent development into embryos. Application of 25mg/l (62.5 μM) for 24 hours was found to increase embryogenesis by more than four fold in the B. napus cultivars Topas and Optima. Similar positive effects of colchicine on embryogenesis have been observed by Iqbal et. al. (1994) with the cultivars Duplo, Janetzki, WP27 and F1 (Janetzki x Duplo). The best results were obtained with colchicine applied at a concentration of 100 mg/l (250 μM) for 24 hours. This treatment was later shown, via flow cytometric analysis, to result in doubling frequencies of 80 to 90% (Möllers et al. 1994). Similarly, in an applied breeding program, Chen et al. (1993) reported that treating microspores with colchicine at a concentration of 0.05 to 0.1% (1.25 to 2.5 mM) for 15 to 22 hours resulted in doubling frequencies in excess of 70%.

Although antimicrotubule herbicides have been used in other crop species for chromosome doubling, only limited exprimental work has been carried out with *B. napus*. To date, only trifluralin has been tested. Eikenberry (1994) reported that microspores treated with trifluralin, applied at 0.5 to 1.0 μM for 24 hours, increased embryogenesis and resulted in a doubling frequency of at least 78.0%. As with colchicine treatments, trifluralin treatment at this concentration did not interfere with plant regeneration or seed set.

In the present study it has been shown that in addition to colchicine and trifluralin, other antimicrotubule agents such as APM, oryzalin and pronamide can

increase the frequency of embryogenesis and chromosome doubling in microspore-derived *B. napus*. Control cultures produced an average of 742 embryos (per 6 x 10^5 plated microspores) and had a spontaneous diploid frequency of 35.5%. With colchicine treatments, the frequency of embryogenesis was only 83.6% that of controls while the doubling frequency was 80.4% of the controls. Since previous studies have suggested that colchicine treatment should increase embryogenesis (Iqbal et al. 1994), the treatment duration of the present experiments may have been too long, or the cultivar tested (Topas) may respond differently to colchicine treatment than other cultivars. With the lower range of concentrations tested, all of the antimicrotubule herbicide treatments had embryo yields superior to that of the control.

Toxicity symptoms, as determined by reduced embryo yield, appeared as herbicide concentration increased. The exception was pronamide which, at the highest concentration of 50 μM, produced the highest embryo yields. One possible explanation for the lesser toxicity of pronamide is that it has slightly different effects on microtubule depolymerization than the other herbicides. Unlike amiprophos-methyl, oryzalin and trifluralin, pronamide does not cause complete depolymerization of microtubules. In root cells of *Allium cepa*, treated with pronamide, remnant microtubule tufts associated with the kinetochores of the chromosomes remain (Vaughan and Vaughn 1987). Incomplete depolymerization may allow microtubule nucleation sites to remain leading to faster recovery of microtubule networks and fewer toxicity symptoms following treatment.

Trifluralin and oryzalin (dinitroaniline herbicides) are closely related chemically. In this study, oryzalin appeared to be slightly more toxic than trifluralin. Trifluralin has

been shown to bind to glass, whereas oryzalin does not (Strachan and Hess 1982). Since glass bottles were used to store stock solutions, it is possible that the actual concentration of trifluralin applied to the microspore cultures may have been lower than originally calculated. Although no tests were carried out to determine if this were the case, this fact could account for the apparent lower toxicity of trifluralin compared to that of oryzalin.

Although antimicrotubule herbicides primarily induce microtubule depolymerization, at higher concentrations they can also increase cytoplasmic calcium concentration by causing passive calcium release from mitochondria and the endoplasmic reticulum (Hertel et. al. 1980). This increased concentration may also contribute to toxicity and reduce the ability of microspores to recover after treatment.

The ability of antimicrotubule agents to increase embryogenesis is believed to be related to the way in which they affect the microtubules of the cytoskeleton which control the location and plane of the cell plate. Microspores, which are embryogenic, are usually in the late uninucleate stage of development and divide symmetrically rather than asymmetrically as seen with normal pollen or gametophytic development. This initial symmetrical division is thought to be a pre-requsite to embryo formation (Zaki and Dickinson 1990). Application of antimicrotubule agents or low temperature can prevent the normal asymmetric division and cause a larger proportion of microspores to divide symmetrically resulting in an increase in the number of microspores which undergo embryogenesis or a sporophytic pathway of development (Zaki and Dickinson 1991).

To induce chromosome doubling and not just increase embryogenesis, treatment should not only inhibit the position where the cell plate forms but also inhibit its

formation during the first embryo division in culture. Treatment duration should therefore extend throughout and slightly beyond this period. In culture, the first embryogenic division of late uninucleate stage microspores of *B. napus* occurs between 8 and 16 hours and for binucleate stage microspores at 8 to 48 hours (Pechan and Keller 1988). In the late uninucleate stage, the chromosome number of microspores has already been doubled via DNA replication. The next step in maintaining this doubled state is to prevent formation of the cell wall during the first embryogenic division. Previous studies have shown that colchicine treatment extending for 24 hours is ideal for this purpose (Zaki and Dickinson 1991, Iqbal et al. 1994). In the present study, microspores were treated for this period of time.

Plant populations regenerated from microspore cultures of *B. napus* contain 10 to 30% spontaneous diploids (Coventry et al. 1988, Chen and Beversdorf 1992). The origin of spontaneous diploids is not fully understood, although it has been suggested that failure of meiosis resulting in unreduced gametes, endomitosis, or even endoreplication may be involved in the process of chromosome doubling (Keller et al. 1975, Heyn 1977, Möllers et al. 1994). It is possible that environmental changes such as temperature, osmotic conditions and other factors in culture, in combination with the proper stage of development, could prevent formation of a cell wall during the first cell cycle in culture. Since late uninucleate microspores have undergone DNA replication, absence of subsequent cell wall formation would result in a cell which is doubled. In effect, this is equivalent to endomitosis.

Antimicrotubule herbicides offer a potential alternative to colchicine as

chromosome doubling agents in *B. napus*. Pronamide in particular, along with trifluralin, were found to produce results superior to that of colchicine. Results from this experiment suggest that five different anti-microtubule agents can be used to increase embryogenesis and chromosome doubling of haploid *B. napus* microspores. Haploid plants (up to 25.0%) which remain after the initial chromosome doubling treatment, will most likely still have to be doubled by the standard method involving the treatment of whole plants with colchicine.

The ability to induce chromosome doubling at the microspore stage of development should result in time, labour, and money savings. The absence of chimaeric plants results in plants with high seed set. The breeder should therefore have enough seed for immediate screening of qualitative and quantitative traits. Similarly, field testing of germplasm can be carried out in the next generation without an extra generation of seed increase. Chromosome doubling of microspores with antimicrotubule agents can therefore be considered to be a significant improvement over previous methods. These techniques should enable a breeder to evaluate a larger number of doubled haploid lines with less time and fewer resources than with colchicine treatment at later stages of development.

Table 3.1.1 Mean percentage values for embryo yield, plant regeneration and diploidization following treatment of *B.napus* microspores with antimicrotubule agents (AMA's).

AMA	Concentration	% Embryo Yield	% Plant Regeneration	% Diploidization
Control	0 µM	100.0 efg	53.8 abc	35.5 f
Colchicine	1.25 mM	83.6 fgh	47.5 bcde	80.4 abc
APM	0.1 µM	121.6 cde	18.8 ef	43.5 ef
	0.5 µM	113.7 def	47.5 bcde	66.6 abcde
	1.0 µM	78.7 gh	53.8 abc	74.4 abcd
	5.0 µM	66.6 ghi	46.3 bcde	71.5 abcde
	10.0 µM	38.0 i	16.3 f	75.0 abcd
	15.0 µM	**		
Oryzalin	0.1 µM	155.5 abc	15.0 f	58.8 abcdef
	0.5 µM	91.1 efgh	28.8 cdef	46.5 def
	1.0 µM	**		
Pronamide	0.1 µM	142.9 bcd	20.0 def	58.8 abcdef
	0.5 µM	172.5 ab	48.8 bcd	48.0 def
	1.0 µM	150.7 abc	47.5 bcde	52.3 bcdef
	5.0 µM	169.3 ab	63.8 ab	65.5 abcde
	10.0 µM	146.6 abcd	28.8 cdef	70.4 abcde
	15.0 µM	150.1 abc	26.3 cdef	82.6 a
	30.0 µM	155.5 abc	77.5 a	73.8 abcd
	50.0 µM	181.4 a	78.8 a	76.3 abcd
Trifluralin	0.05 µM	162.2 ab	67.5 ab	50.8 cdef
	0.1 µM	163.1 ab	45.0 bcde	65.8 abcde
	0.5 µM	100.5 efg	27.5 cdef	79.7 abc
	1.0 µM	114.3 def	46.3 bcde	83.9 a
	5.0 µM	63.9 hi	27.5 cdef	81.9 ab
	10.0 µM	**		

* Embryo percentages are calculated as a percentage of the control in which an average of 742.0 embryos per $6x10^5$ plated microspores were produced. Results are based on four replicates.

** Concentration beyond which embryogenesis ceased to occur.

Values which share the same letter(s) are not significantly different from each other (@ P= 0.05) according to Duncan's multiple range test.

The pooled standard error values were ±78.9 embryos per $6x10^5$ plated microspores or ±10.6% (embryo yield), ±8.7% (plant regeneration) and ±8.8% (diploidization).

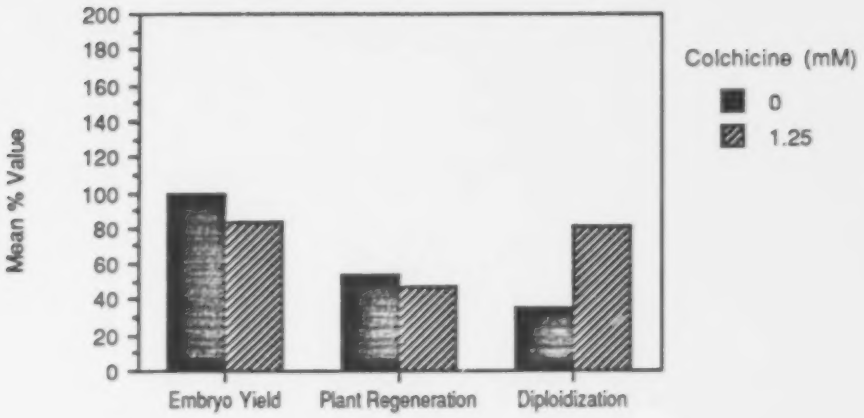

Figure 3.1.1 Effect of colchicine treatment of microspores on subsequent embryo yield, plant regeneration, and diploidization in *B. napus*. (Pooled SE= ±10.6, 8.7 and 8.8% for embryo yield, plant regeneration and diploidization respectively).

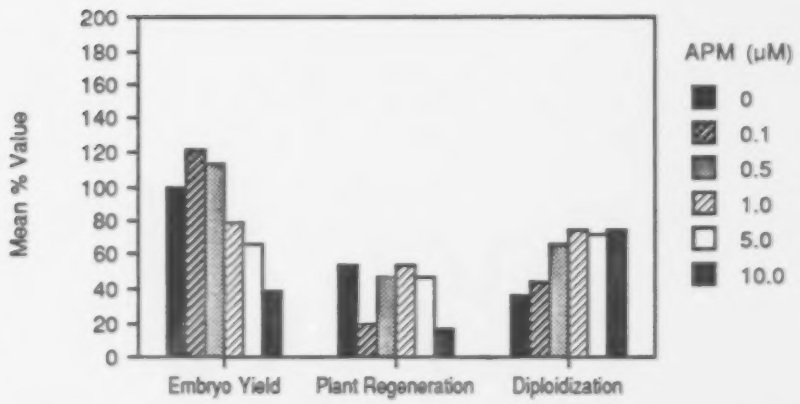

Figure 3.1.2 Effect of amiprophos-methyl treatment of microspores on subsequent embryo yield, plant regeneration, and diploidization in *B. napus*. (Pooled SE= ±10.6, 8.7 and 8.8% for embryo yield, plant regeneration and diploidization respectively).

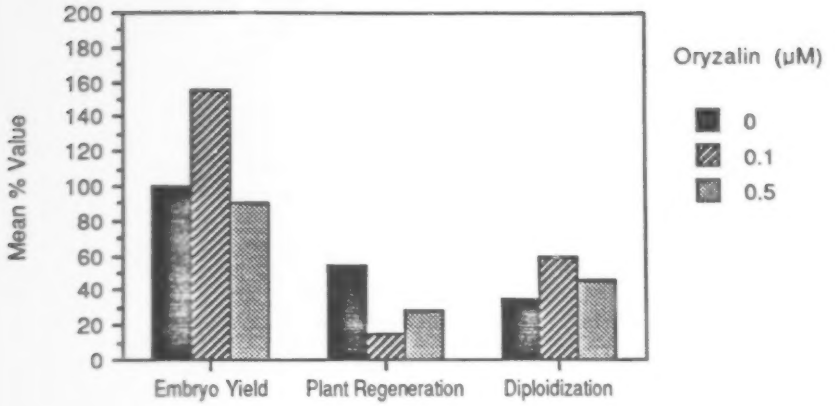

Figure 3.1.3 Effect of oryzalin treatment of microspores on subsequent embryo yield, plant regeneration, and diploidization in *B. napus*. (Pooled SE= ±10.6, 8.7 and 8.8% for embryo yield, plant regeneration and diploidization respectively).

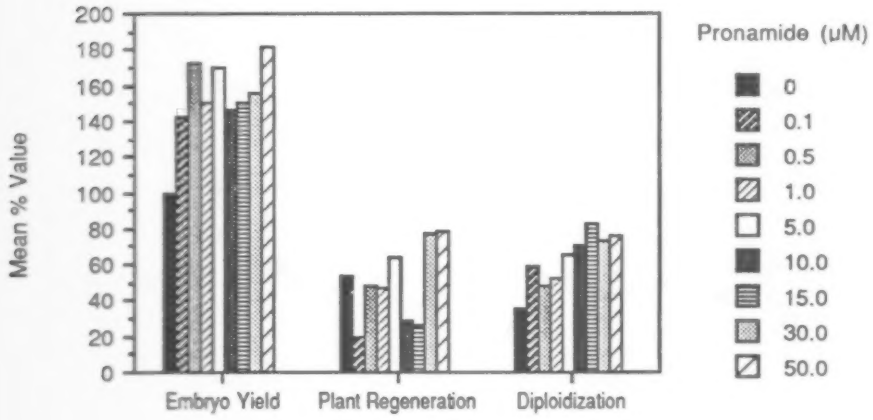

Figure 3.1.4 Effect of pronamide treatment of microspores on subsequent embryo yield, plant regeneration, and diploidization in *B. napus*. (Pooled SE= ±10.6, 8.7 and 8.8% for embryo yield, plant regeneration and diploidization respectively).

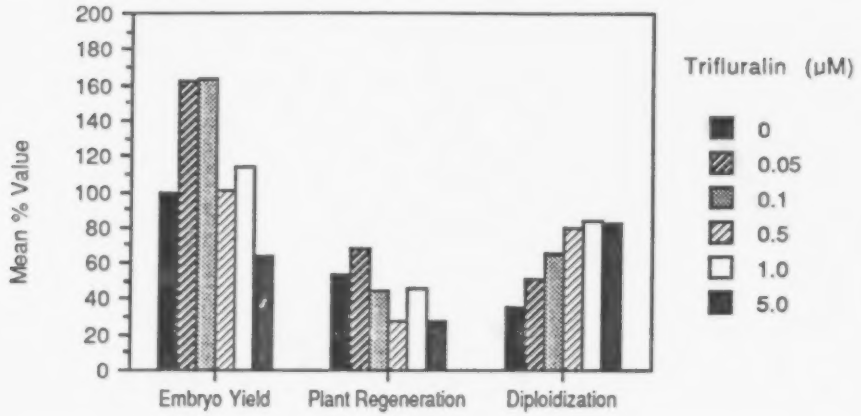

Figure 3.1.5 Effect of trifluralin treatment of microspores on subsequent embryo yield, plant regeneration, and diploidization in *B. napus*. (Pooled SE= ±10.6, 8.7 and 8.8% for embryo yield, plant regeneration and diploidization respectively).

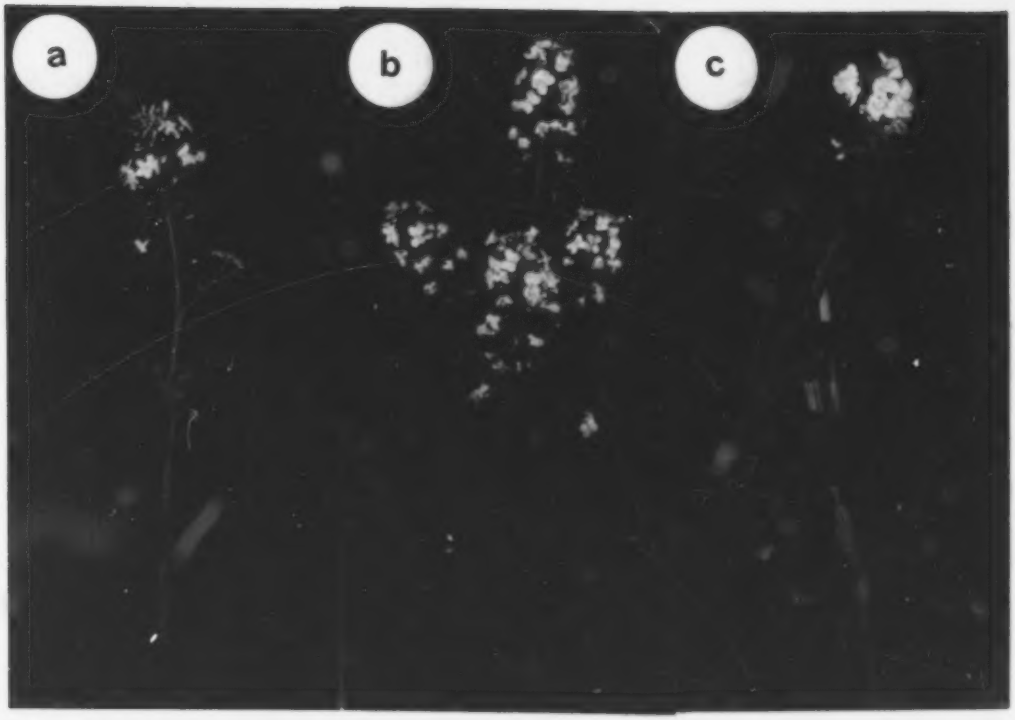

Figure 3.1.6 Appearance of a) haploid, b) diploid, and c) tetraploid microspore-derived *B. napus* L. cv. Topas.

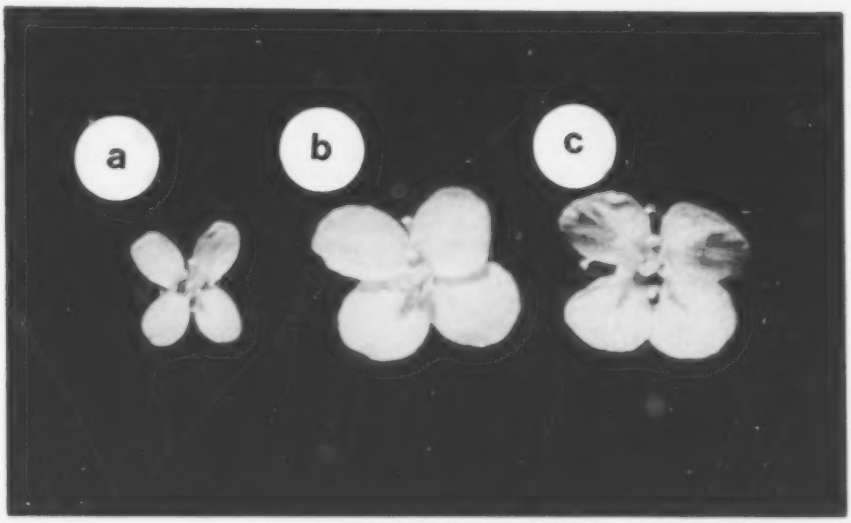

Figure 3.1.7 Flower morphology of a) haploid, b) diploid, and c) tetraploid microspore-derived *B. napus* L. cv. Topas.

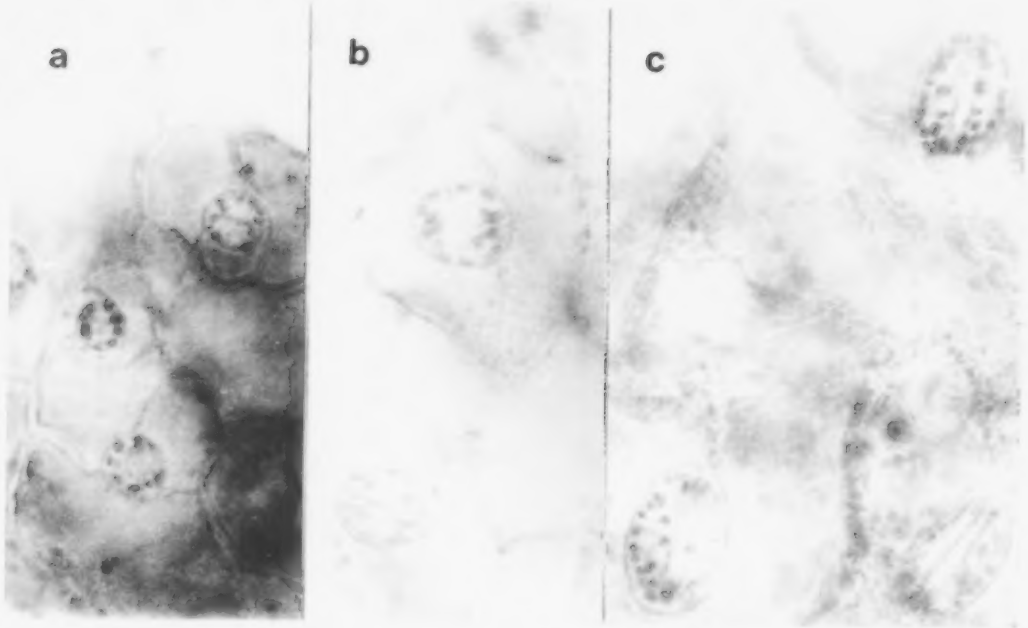

Figure 3.1.8 Guard cell chloroplasts from leaves of a) haploid, b) diploid, and c) tetraploid *B. napus* L. cv. Topas (Magnification= 830x).

Figure 3.1.9 Chromosomes from *B. napus* L. cv. Topas a) haploid (2n=2x=19)(diplotene), b) diploid (2n=4x=38)(diakinesis), and c) tetraploid (1n=4x=38) pollen mitosis I (late prophase) (Magnification= 1600x).

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3.2 Experiment #2: Chromosome Doubling of Microspore-derived Embryos in *Brassica napus* L. cv. Topas by Antimicrotubule Agents.

3.2.1 Abstract

Microspore-derived embryos of *Brassica napus* L. (c.v. Topas) were treated with amiprophos-methyl, oryzalin, pronamide, trifluralin and colchicine in order to evaluate the effectiveness of chromosome doubling treatments. Thirty-day old embryos were treated with concentrations of 0.1, 1.0 and 10.0 μM for periods of 1, 2 and 4 days. Diploidization frequencies tended to increase as herbicide concentration increased but usually did not exceed 50%. Oryzalin and trifluralin treatments, 10.0 μM for 4 days, produced diploidization frequencies of 78.6% and 61.1% respectively. These frequencies exceeded or equaled chromosome doubling frequencies obtained with colchicine treatment of whole plants and plantlets.

3.2.2 Introduction

Microspore culture is an efficient technique used by *B. napus* breeders for producing large numbers of haploid plants. When the chromosome number of haploids are doubled a homozygous line is produced. These lines can then be selected for various quantitative and quality traits important to plant breeders. Although homozygous lines are rapidly developed with this method, deficiencies exist with procedures used to produce them.

Chromosome doubling of microspore-derived haploid plants has traditionally involved treating whole plants with the antimicrotubule chemical, colchicine. This

alkaloid, extracted from the autumn crocus (*Colchicum autumnale*), selectively binds to α and β-tubulin subunits which normally polymerize to form plant microtubules (Hart and Sabnis 1976; Andreu and Timasheff 1986; Hassawi and Liang 1991). Consequently, processes which require functioning microtubules, such as chromosome movement and cell division, are inhibited. Since colchicine can effectively prevent cell division without inhibiting the chromosome cycle, it has been utilized for many years as an effective agent for chromosome doubling of plant cells (Blakeslee and Avery 1937; Levan 1938). Treatment of haploid *B. napus* plants with colchicine results in an excess of more than 60% chromosome doubling amongst treated plants (Gland 1981; Coventry et al. 1988). However, this treatment is lacking in efficiency. Treated plants may require a period of up to 3 months to recover, form tissues that are sectorial chimeras, produce only one doubled shoot and have poor seed set. A further generation of seed increase is usually required to obtain useful quantities of seed for laboratory analysis and field evaluation.

To overcome the inefficiencies associated with colchicine treatment of whole plants it is essential to investigate the effectiveness of applying treatment of colchicine and other antimicrotubule agents at earlier stages of development. Amiprophos-methyl (APM), oryzalin, pronamide and trifluralin are herbicides which bind to tubulin subunits and inhibit their polymerization into microtubules. Unlike colchicine, which is effective at mM concentrations, these agents are effective at μM concentrations (Morejohn and Fosket 1991; Vaughn and Lehnen 1991). The main objective of this experiment was to evaluate the chromosome doubling effectiveness of these antimicrotubule herbicides and colchicine when applied to 30 day old microspore-derived embryos of *B. napus* cv. Topas.

3.2.3 Materials and Methods

Growth of Microspore Donor Plants

Procedures followed for growing donor plants and microspore culture were those of (Coventry et. al 1988) and (Swanson 1990). Seeds of *B. napus* L. cv. Topas were germinated at 18^{0}C on moist filter paper in petri dishes. Seedlings were transferred to 6-inch pots containing metro mix (W.R. Grace & Co. Canada) soil-free growth media. The pots were placed in a growth cabinet maintained at 24/21^{0}C day/night temperature with a 16 hour photoperiod and photon flux density of 425-450 μmol $m^{-2}s^{-1}$. At the four-leaf stage, the number of plants were thinned to one per pot. Plants were watered and fertilized as required, 3 to 4 times weekly, with a solution containing 1 g/L of (20-20-20 [N:P:K]) fertilizer. As the first buds began to form, the temperature in the growth cabinet was reduced to 10/5^{0}C day/night.

Microspore Isolation

Flower buds, 1.5-3.5 mm in length, were surface sterilized in a solution of sodium hypochlorite 7.0% (w/v) for 10 minutes and rinsed three times for 5 minutes with sterile ice cold water. The buds were gently crushed in a mortar and pestle with B5 isolation medium containing 13% (w/v) sucrose (Appendix A) (Lichter 1982), and the homogenate filtered through a 63 and 44 μm nylon mesh (Nytex). The filtrate was then poured into a 50 mL Falcon tube (Fisher Scientific, Canada) and centrifuged at 250g for 10 minutes. The microspore pellet was washed and centrifuged twice with B5 medium and resuspended in modified NLN medium containing 13% sucrose (Appendix B) (Lichter

1982). The microspore concentration was adjusted, with a haemocytometer, to approximately 6x 10^4 microspores/mL. Aliquots of 10 mL were dispensed in (100x 15mm) petri dishes, sealed with parafilm and placed in an incubator at 30 ^{0}C in the dark. After 14 days, the plates were placed on a rotary shaker (60 rpm) at room temperature in the dark.

Herbicide Stock Solutions

Amiprophos-methyl (APM) was donated by the Mobay Chemical Corporation. (Kansas City, U.S.A.), pronamide from Rohm and Haas Co. (Philadelphia, U.S.A.), and oryzalin and trifluralin from Dow Chemical (Midland, U.S.A.). The antimicrotubule herbicides, and colchicine (Fisher Scientific, Canada), were dissolved in acetone to produce stock solutions with a final concentration of 4 mM.

Herbicide Treatment

The herbicide and colchicine stock solutions were added to 5.0 mL of NLN culture media in (50x 15mm) petri dishes, to produce concentrations of 0.1, 1.0, and 10.0 μM. Groups of 100 to 200 cotyledonary embryos at 30 days of development, were placed in the petri dishes. The petri dishes were wrapped in parafilm and placed on a slow (50-60 rpm) rotary shaker, in the dark, for periods of 1, 2, and 4 days. After treatment, the embryos were washed three times with NLN culture medium and transferred to petri dishes (20 embryos per plate) containing solid B5 culture medium supplemented with 0.15 mg/L gibberelic acid (Appendix C). The plates were incubated for 10 days at 4^{0}C, with

an 8 hour photoperiod at a photon flux density of 30 μmol $m^{-2}s^{-1}$. Subsequently, the plates were incubated at room temperature (24°C), with a 12 hour photoperiod at a photon flux density of 30 μmol $m^{-2}s^{-1}$, until the embryos germinated and formed true leaves (30-40 days). The numbers of surviving plants per treatment were recorded.

Plantlet Transfer and Plant Regeneration

Plantlets (germinated embryos) were transferred to multiwell transfer flats containing moist soil-free mix (metro mix). Plantlets were watered, misted and covered with clear plastic to maintain humidity. After one week, the plastic was removed and the plantlets irrigated 3 to 4 times weekly with a solution containing 1 g/L of (20-20-20 [N:P:K]) fertilizer.

Ploidy Determination

Plant ploidy level was determined by examining individual shoots for flower morphology and the ability of flowers to produce pollen (Keller and Armstrong 1978; Siebel and Pauls 1989; Mathias and Robbelen 1991). Haploid plants produced buds which atrophied, anthers which were devoid of pollen, and did not set seed. Diploid plants produced larger flower buds, anthers which produced pollen abundantly, and set seed. Mixoploids produced both haploid and diploid shoots. Tetraploid plants had large buds and produced pollen abundantly, but formed only a few pods containing seeds.

Statistical Analysis

The experiment consisted of two replicates of 20 transferred embryos for each treatment; 40 embryos per treatment were transferred. The means of the various treatments were compared using Duncan's multiple range test (Steel and Torrie 1980).

3.2.4 Results

Plant Population Regenerated

A total of 941 plants were regenerated from all of the treatments. Of these plants, 595 (63.2%) were haploid, 312 (33.2%) diploid, 23 (2.4%) mixoploid (haploid/diploid), and 11 (1.2%) tetraploid.

Effect of Anti-microtubule Agents on Plant Regeneration

The comparison of means (Duncan's multiple range test) (Table 3.2.1), showed that regeneration frequencies of 20.0% or less were significantly different, or worse, than the control regeneration frequency. No treatments had regeneration frequencies that were significantly better than that of the control.

The average regeneration frequency of the control was 67.5%. The highest regeneration frequency was 97.5% for colchicine, 10 μM for 4 days, followed by 87.5% for trifluralin, 10 μM for 2 days. The only treatments which produced regeneration frequencies that were significantly less than the control were pronamide, 1.0 μM for 1 day, and oryzalin, 1.0 μM for 4 days, at 20.0% and 12.5% respectively. Regeneration data for APM (Figure 3.2.1), oryzalin (Figure 3.2.3), pronamide (Figure 3.2.5), trifluralin

(Figure 3.2.7), and colchicine (Figure 3.2.9) did not suggest increased concentration or duration caused a decline in regeneration of embryos into plants. The herbicide concentrations used in this experiment were not high enough to produce visible toxicity symptoms in treated embryos.

Effect of Anti-microtubule Agents on Diploidization

The comparison of means (Duncan's multiple range test) (Table 3.2.1), suggested that only oryzalin, 10.0 μM for 4 days, with a diploidization frequency of 78.6%, was significantly better than the control value of 23.0%.

APM diploidization frequencies ranged from 10.0%, 0.1 μM for 4 days, to 50.0%, 10.0 μM for 2 or 4 days. Oryzalin frequencies ranged from a low of 20.0%, 10.0 μM for 1 day, to a high of 78.6%, 10.0 μM for 4 days. Pronamide treatment resulted in a low of 17.5%, 1.0 μM for 4 days, and a high of 40.0%, 10.0 μM for 4 days. Trifluralin had a low of 19.8%, 0.1 μM for 4 days, and high of 61.1%, 10.0 μM for 4 days. Colchicine treatment produced a low of 11.7%, 10.0 μM for 2 days, and high of 41.7%, 1.0 μM for 1 day. Diploidization data for APM (Figure 3.2.2), oryzalin (Figure 3.2.4), pronamide (Figure 3.2.6), trifluralin (Figure 3.2.8), and colchicine (Figure 3.2.10) shows that most treatments produced superior results to the control. With oryzalin and trifluralin it appears that chromosome doubling increases as concentration and duration increase.

Most treatments generally resulted in doubling frequencies of less than 50.0%. Only trifluralin, 10.0 μM for 4 days, @ 61.1%, and oryzalin, 10.0 μM for 4 days, @ 78.6%, exceeded 60.0% diploidization, which is the level of diploidization reported for

colchicine treatment of whole plants or plantlets (3-4 leaf stage) (Gland 1981; Coventry et al. 1988; Mathias and Röbbelen 1991).

Mixoploids

Only 23 (2.4%) out of the 941 plants regenerated were mixoploids having both haploid and diploid shoots. Since only the first formed shoots of mature plants were used to determine ploidy level, this value may be an underestimate of the actual numbers of plants which were mixoploids. The mixoploid plants were considered to be diploidized in calculating the diploidization frequencies.

Autotetraploids

Only 11 (1.2%) of the plants regenerated were determined to be tetraploid. Tetraploid plants formed larger flower buds, thicker stems and leaves, and had larger leaf veins than diploid plants. The flowers of these plants had larger anthers and carpels, and broader more deeply pigmented petals than diploid flowers.

Seed Set

The seed set of most regenerated plants was not recorded since most plants were discarded after the ploidy level was determined. Of the few regenerated diploids allowed to mature, seed set was similar to seed-derived plants. Mixoploids, which were allowed to mature, often had single shoots with moderate seed set, but also formed shoots which produced only a few pods with poor seed set.

3.2.5 Discussion

Microspore-derived plant populations in *B. napus* have been reported to have a spontaneous diploidization frequency ranging from approximately 10 to 30% (Coventry et al. 1988, Chen and Beversdorf 1992). This frequency is somewhat low for use in a breeding program, and most plants have to be treated with colchicine for chromosome doubling during the early stages of flowering. Colchicine treatment at this stage is inefficient due to relatively slow recovery of treated plants, incomplete doubling of shoots, poor seed set, and the requirement of an extra generation of seed increase.

To improve chromosome doubling techniques, researchers have attempted to apply treatment at earlier stages of development. Application of 50 mg/L (125 μM) colchicine for 4 to 8 days, at the early plantlet (3-4 leaf) stage of development, has been shown to be highly effective for chromosome doubling in *B. napus* (Mathias and Röbbelen 1991). Although the chromosome doubling frequency was similar to that seen with whole plant treatment (60%), the quality of plants was shown to be much better. Plantlet treatment resulted in plants that were of one ploidy level, lacking chimaeric tissues, and had relatively high seed set. Consequently, an extra generation for seed increase was not required. Colchicine treatment at the microspore stage has proven to be even more effective than treatment of plantlets. Chromosome doubling frequencies of approximately 80% have been routinely obtained using concentrations of 0.025 to 0.1% (625 μM to 2.5 mM) applied for 24 hours (Chen et al. 1993; Möllers et al. 1994). In addition, colchicine treatment of microspores has been shown to increase embryogenesis and have few toxic side effects on most genotypes tested (Chen et al. 1993; Möllers et al. 1994).

As an alternative to colchicine, antimicrotubule herbicide treatments have been applied to haploid plant material. These agents affect microtubules and the processes they mediate in a fashion similar to colchicine. However, they induce these effects at μM concentrations rather than the mM concentrations required for colchicine (Morejohn and Fosket 1991; Vaughn and Lehnen 1991).

Wan et al. (1991) treated haploid maize-derived callus with APM, oryzalin, pronamide and trifluralin. Treatments of APM (15 μM applied for 3 days) and pronamide (5 μM applied for 3 days) produced regeneration frequencies in excess of 50.0% and doubling frequencies of 67 and 73.5% respectively. Oryzalin and trifluralin, although able to induce chromosome doubling, inhibited callus growth and plant regeneration at effective concentrations. Hassawi and Liang (1991) have also tested trifluralin, oryzalin and colchicine on anther-derived haploid calli of wheat. Oryzalin and trifluralin (10 μM applied for 3 days) produced doubling frequencies in excess of 50.0% in the cultivar "Pavon", but were not effective in the cultivar "Kitt". Colchicine treatment (313 to 626 μM applied for 2 to 3 days) produced diploidization frequencies of 88.9% in Pavon and 42.9% in Kitt, and were superior to oryzalin and trifluralin treatments.

In *B. napus*, trifluralin has been applied at the microspore stage of development (Eikenberry 1994). Treatment of freshly isolated microspores with trifluralin (0.5 to 1.0 μM for 24 hours) induced doubling frequencies in excess of 78.0%. As with colchicine treatment of microspores, trifluralin treatment also resulted in increased embryogenesis of cultures.

The present study illustrates the varying efficiencies anti-microtubule agents have

for inducing chromosome doubling in 30 day old microspore-derived embryos of *B. napus* cv. Topas. None of the agents tested appeared to induce post-treatment toxicity effects. Colchicine, as a comparison, was applied at the same concentrations (0.1 to 10 μM) as the antimicrotubule herbicides. For chromosome doubling, colchicine concentrations of 125 μM and higher are more effective (Mathias and Röbbelen 1991). Low levels of colchicine can stimulate plant growth, whereas higher concentrations that affect cell division, can be inhibitory to plant growth (Walker 1938). The highest level and longest duration for colchicine treatment in this experiment (10.0 μM for 4 days) also produced the highest plant regeneration frequency. This concentration is more than 100 fold less than the concentration of colchicine normally used to double the chromosome number in *B. napus*, and therefore, may be within the concentration range effective for stimulating plant growth. Possibly, application of such a treatment for increasing embryo germination could be developed. Additional testing is required to determine if this approach is feasible.

Treatment of embryos with antimicrotubule agents did not always produce plants which were completely doubled or of one ploidy level, as has been reported previously for colchicine treatment of plantlets at the 3 to 4 leaf stage (Mathias and Röbbelen 1991). Since the meristematic regions of the embryos are composed of a number of cells, it appears that in some cases sectorial chimaeras occurred in which only a few meristematic cells were doubled, while others remained haploid. Tetraploids which were produced most likely result from chromosome doubling of embryos which were already spontaneous diploids. Since mixoploids and tetraploids occurred in relatively few cases, 2.4 and 1.2%

of the total population respectively, they may not have a large impact on the overall efficiency of chromosome doubling. In contrast to treatment of embryos, it has been shown that treatment of microspores with colchicine or trifluralin produces plants of only one ploidy level and diploids having excellent seed set (Eikenberry 1994; Möllers et al. 1994). Because of decreased seed set in mixoploid plants, chromosome doubling at the embryo stage of development may not be as efficient as treatment at the microspore stage of development.

Chromosome doubling tended to increase with increased concentration and with treatment duration. Most treatments resulted in doubling frequencies of less than 50.0%. Only trifluralin (10.0 μM for 4 days), @ 61.1%, and oryzalin (10.0 μM for 4 days), @ 78.6%, exceeded 60.0% diploidization. This later value has been obtained with whole plants (Gland 1981; Coventry et al. 1988), and plantlets (2-4 leaf stage) (Mathias and Röbbelen 1991) treated with colchicine. However, in comparison to treatment of microspores, in which more than 80% doubling occurred, treatment of 30 day embryos is still less efficient.

Table 3.2.1 Mean percentage values for plant regeneration and diploidization of 30-day old microspore-derived *B. napus* embryos treated with various antimicrotubule agents, concentrations and periods of duration.

AMA	Concentration	Treatment Duration	% Plant Regeneration	% Diploidization
Control	0 µM	0D	67.5 abcde	23.0 cde
APM	0.1 µM	1D	57.5 abcdefg	47.7 abcd
		2D	55.0 abcdefg	40.9 bcde
		4D	45.0 bcdefg	10.0 e
	1.0 µM	1D	45.0 bcdefg	33.3 bcde
		2D	25.0 efg	43.8 abcde
		4D	50.0 bcdefg	29.7 bcde
	10.0 µM	1D	55.0 abcdefg	33.3 bcde
		2D	35.0 defg	50.0 abc
		4D	30.0 defg	50.0 abc
Oryzalin	0.1 µM	1D	55.0 abcdefg	36.7 bcde
		2D	47.5 bcdefg	42.3 bcde
		4D	35.0 defg	43.8 abcde
	1.0 µM	1D	37.5 cdefg	20.8 cde
		2D	60.0 abcdef	42.0 bcde
		4D	12.5 g	58.3 ab
	10.0 µM	1D	27.5 efg	20.0 cde
		2D	75.0 abcd	36.7 bcde
		4D	32.5 defg	78.6 a
Pronamide	0.1 µM	1D	67.5 abcde	39.8 bcde
		2D	32.5 defg	29.8 bcde
		4D	70.0 abcde	39.3 bcde
	1.0 µM	1D	20.0 fg	33.3 bcde
		2D	55.0 abcdefg	30.8 bcde
		4D	67.5 abcde	17.5 cde
	10.0 µM	1D	30.0 defg	18.2 cde
		2D	70.0 abcde	39.3 bcde
		4D	82.5 abc	40.0 bcde

AMA	Concentration	Treatment Duration	% Plant Regeneration	% Diploidization
Trifluralin	0.1 µM	1D	35.0 defg	35.6 bcde
		2D	35.0 abcdef	35.4 bcde
		4D	62.5 abcdef	19.8 cde
	1.0 µM	1D	57.5 abcdefg	51.9 abc
		2D	62.5 abcdef	40.0 bcde
		4D	75.0 abcd	40.2 bcde
	10.0 µM	1D	35.0 defg	47.8 abcd
		2D	87.5 ab	51.7 abc
		4D	67.5 abcde	61.1 ab
Colchicine	0.1 µM	1D	30.0 defg	34.2 bcde
		2D	25.0 efg	12.5 de
		4D	82.5 abc	12.4 e
	1.0 µM	1D	30.0 defg	41.7 bcde
		2D	65.0 abcdef	38.2 bcde
		4D	60.0 abcdef	34.3 bcde
	10.0 µM	1D	27.5 efg	19.6 cde
		2D	75.0 abcd	11.7 e
		4D	97.5 a	33.4 bcde

Treatment values are based on the results of two replicates of twenty transferred embryos each.

Values followed by the same letter(s) are not significantly different (@ P= 0.05) according to Duncan's multiple range test.

The overall pooled error values were ±12.9 (plant regeneration) and ±12.4 (diploidization).

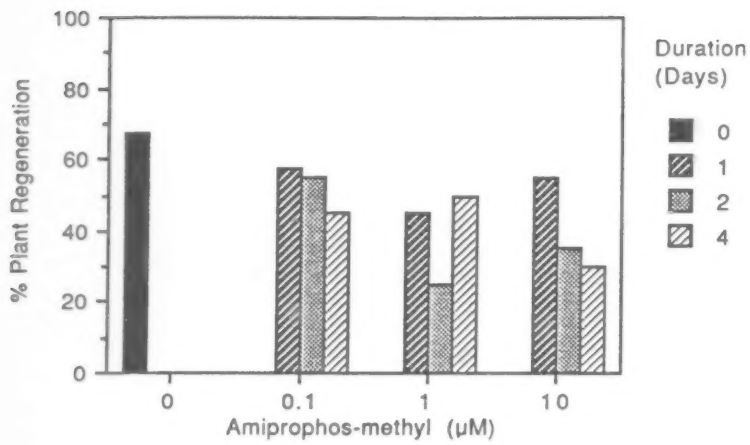

Figure 3.2.1 Effect of amiprophos-methyl treatment of microspore-derived *B. napus* embryos on subsequent plant regeneration. (Pooled SE= ±12.9%).

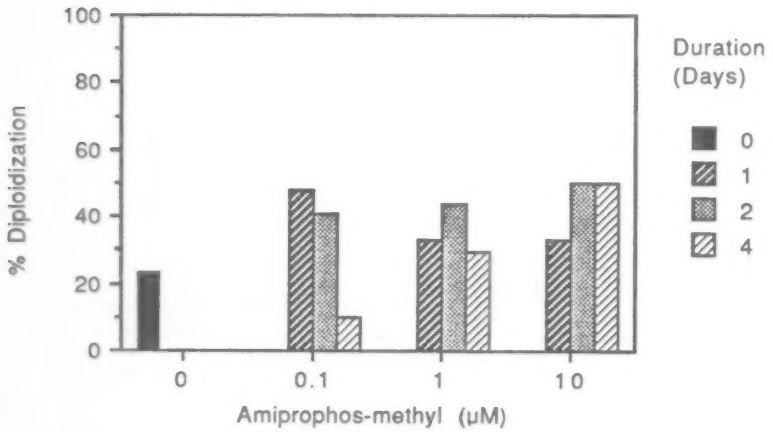

Figure 3.2.2 Effect of amiprophos-methyl treatment of microspore-derived *B. napus* embryos on subsequent diploidization. (Pooled SE= ±12.4%).

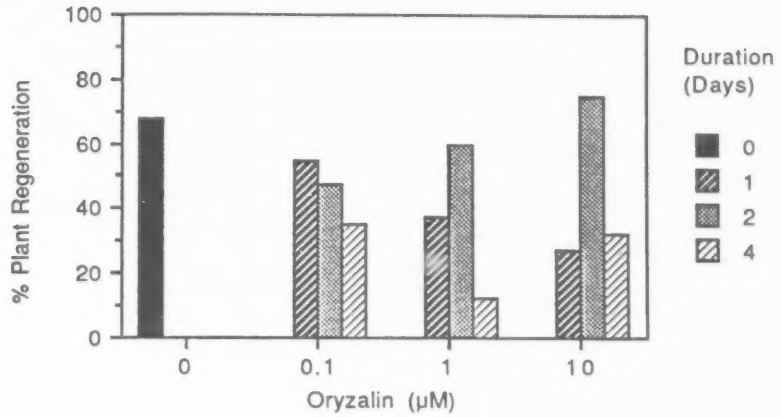

Figure 3.2.3 Effect of oryzalin treatment of microspore-derived *B. napus* embryos on subsequent plant regeneration. (Pooled SE= ±12.9%).

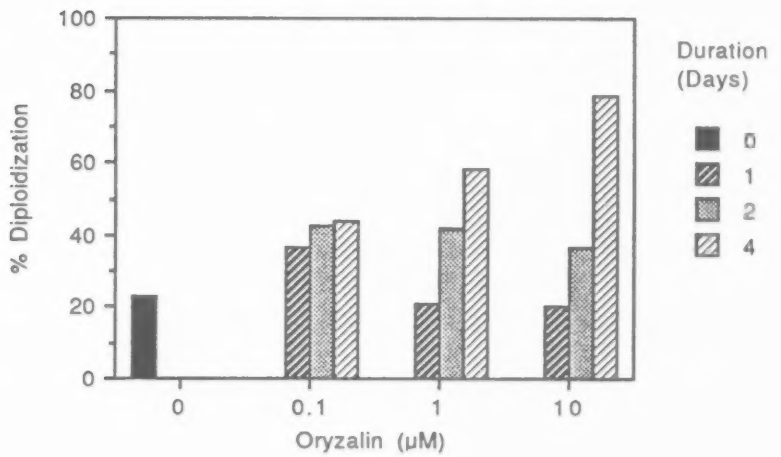

Figure 3.2.4 Effect of oryzalin treatment of microspore-derived *B. napus* embryos on subsequent diploidization. (Pooled SE= ±12.4%).

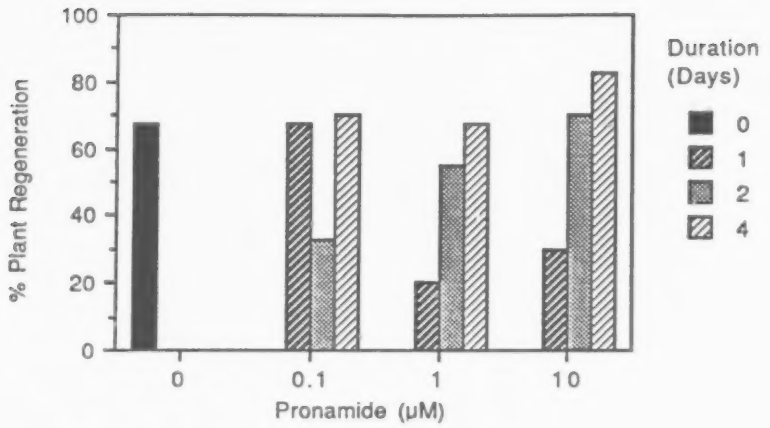

Figure 3.2.5 Effect of pronamide treatment of microspore-derived *B. napus* embryos on subsequent plant regeneration. (Pooled SE= ±12.9%).

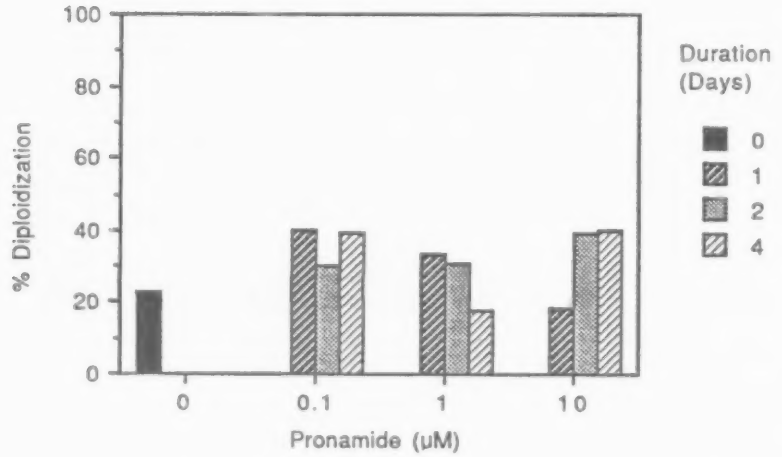

Figure 3.2.6 Effect of pronamide treatment of microspore-derived *B. napus* embryos on subsequent diploidization. (Pooled SE= ±12.4%).

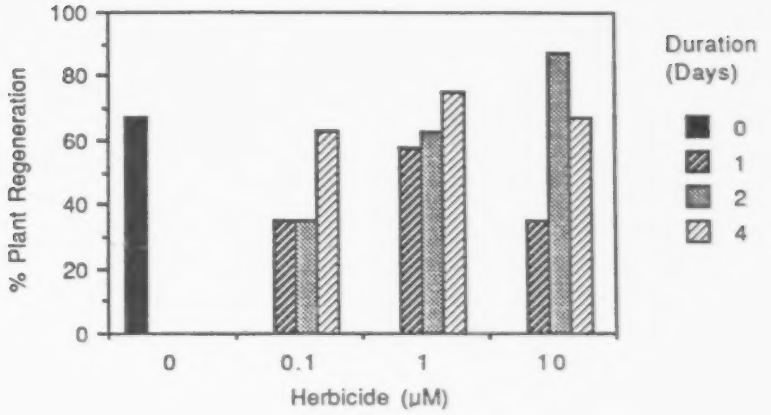

Figure 3.2.7 Effect of trifluralin treatment of microspore-derived *B. napus* embryos on subsequent plant regeneration. (Pooled SE= ±12.9%).

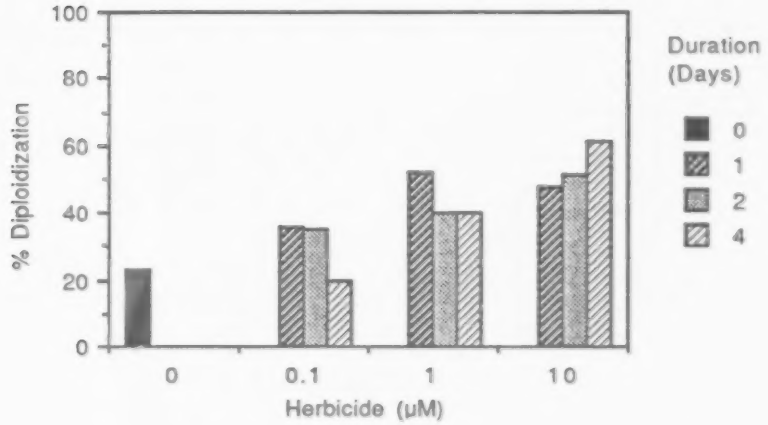

Figure 3.2.8 Effect of trifluralin treatment of microspore-derived *B. napus* embryos on subsequent diploidization. (Pooled SE= ±12.4%).

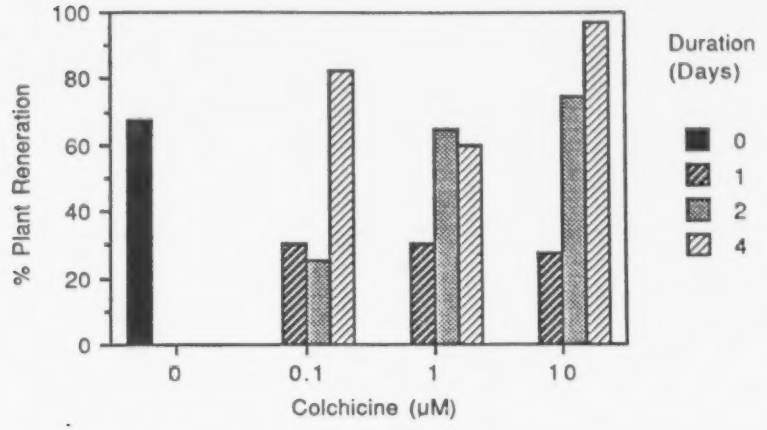

Figure 3.2.9 Effect of colchicine treatment of microspore-derived *B. napus* embryos on subsequent plant regeneration. (Pooled SE= ±12.9%).

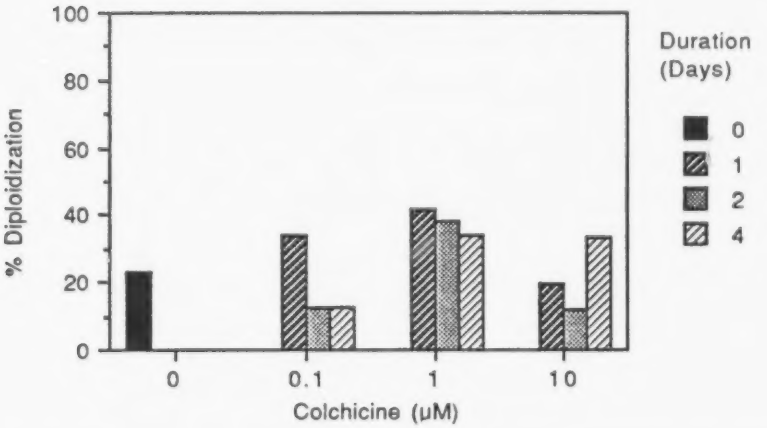

Figure 3.2.10 Effect of colchicine treatment of microspore-derived *B. napus* embryos on subsequent diploidization. (Pooled SE= ±12.4%).

3.2.6 Literature Cited

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3.3 Experiment #3: Cryo-induced Chromosome Doubling of Microspores in *Brassica napus* L. cv. Topas: cryo-treatment of microspores from donor plants grown at reduced temperature.

3.3.1 Abstract

A simple cryo-treatment procedure was developed to produce doubled haploid microspore-derived plants in *Brassica napus* L. cv. Topas. Isolated microspores were cooled at a standard rate (0.5°C/minute) to various terminal temperatures before immediate thawing, or thawing after liquid nitrogen immersion. Treated samples were subsequently cultured and the effects of terminal temperature and liquid nitrogen immersion on embryo yield, plant regeneration, and diploid plant production were analysed. Embryo production decreased as terminal temperatures decreased. Liquid nitrogen immersion was effective only when samples reached terminal temperatures of -45 and -60°C. Liquid nitrogen immersion resulted in a decrease in embryo yield compared to non-immersed samples, whereas, plantlet germination increased in liquid nitrogen immersed samples. Diploidization frequencies increased as terminal temperature decreased. Liquid nitrogen immersion did not affect the diploidization frequency beyond that obtained with cooling. A maximum value of 87.5% diploids occurred at the lowest terminal temperature of -60°C. Cryo-treatment of microspores by cooling at -0.5°C/minute to -60°C is an effective method for inducing chromosome doubling in microspore-derived plant populations of *B. napus* cv. Topas. In combination with liquid nitrogen storage, this treatment can be used for both long term storage and doubled haploid production.

3.3.2 Introduction

Many *B. napus* breeding programs now routinely employ microspore culture as a rapid and efficient means for producing homozygous doubled haploid lines within a single generation. Rapid evaluation of important biochemical and agronomic traits can thus take place in the following generation resulting in a considerable increase in breeding efficiency. In a conventional breeding program, up to ten years may be required before inbred homozygous lines are produced and field evaluated.

As efficient as current microspore culture techniques are, there are still many aspects of the technique which can be improved. In breeding programs, one of the main difficulties following initial generation of haploid plants, is inducing chromosome doubling. Haploid plants have traditionally been doubled by treatment at the mature (flowering) stage with colchicine. In this procedure, roots are trimmed and the roots of the trimmed plants subsequently placed in a colchicine solution which is absorbed and translocated to the shoot meristematic regions. Although this treatment results in more than 60% of treated plants producing at least one or more doubled shoots, it is somewhat crude. Plants tend to die back and can require approximately three months for recovery. Doubling is often incomplete with formation of sectorial chimaeras that have reduced seed set relative to normal diploid plants. An additional generation is often required before sufficient seed is produced for chemical analysis and field evaluation of lines. To overcome these difficulties it is desirable to investigate alternative methods of chromosome doubling that produce fewer toxic side effects, and can be applied at earlier stages of development.

Charne et al. (1988) showed that cryo-treating isolated microspores significantly increased the frequency of spontaneous diploids in populations of microspore-derived plants. Although, early experimental work demonstrated that the frequency of chromosome doubling could be increased by cryo-treatment, a significant reduction in survival and subsequent embryogenesis of microspores was observed. Significant improvements in survival rates were recently made by Chen and Beversdorf (1992b).

The main objectives of the present study were to determine the effects of terminal temperatures (-15, -30, -45 and -60^{0}C) and liquid nitrogen immersion on microspore embryogenesis and diploidization. Unlike previous cryo-experiments, microspore donor plants in this experiment were grown at low temperature to produce an initial microspore population which was more embryogenic and more acclimated to low temperatures. It was hoped that by using more embryogenic and viable starting material a cryopreservation protocol which results in higher rates of survival and diploidization could be developed.

3.3.3 Materials and Methods

Growth of Microspore Donor Plants

Procedures followed for growing donor plants and microspore culture were those of (Coventry et. al 1988) and (Swanson 1990). Seeds of *B. napus* L. cv. Topas were germinated at 18^{0}C on moist filter paper in petri dishes. Seedlings were transferred to 6-inch pots containing metro mix (W.R. Grace & Co. Canada) soil-free growth media. The pots were placed in a growth cabinet maintained at 24/21^{0}C day/night temperature with a 16 hour photoperiod and photon flux density of 425-450 μmol $m^{-2}s^{-1}$. At the

four-leaf stage, the plants were thinned to one per pot. Plants were watered as required, 3 to 4 times weekly, with a solution containing 1 g/L of (20-20-20 [N:P:K]) fertilizer. As the first buds began to form the temperature in the growth chamber was reduced to 10/5^{0}C day/night.

Microspore Isolation

Buds at the proper stage of development (1.5-3.5 mm in length) were excised from donor plants and placed on ice for a short period of time (< 30 min.) before isolation. Buds were surface sterilized in a solution of sodium hypochlorite 7.0% (w/v) for 10 minutes and rinsed three times for 5 minutes with sterile water. The buds were gently crushed in a mortar and pestle with B5 isolation medium containing 13% (w/v) sucrose (Appendix A) (Lichter 1982), and the homogenate filtered through 63 and 44 μm nylon mesh (Nytex). The filtrate was poured into a 50 mL Falcon tube (Fisher Scientific, Canada) and centrifuged at 250g for 10 minutes. The microspore pellet was washed and centrifuged twice with B5 medium. The microspores were resuspended in modified NLN medium containing 13% sucrose (Appendix B) (Lichter 1982) and adjusted, with a haemocytometer, to a final concentration of approximately 1x 10^6 microspores/mL. Microspore isolation steps were carried out at reduced temperature (0 to 5^{0}C). Samples (0.6 mL) were placed into 1 mL cryovials and allowed to equilibrate on ice (0^{0}C) for 2 hours.

Cryo-treatment

Microspore samples were placed in a cryo-cooler (Figure 3.3.4) connected to a programable temperature controller, Omega series CN-2010 (Omega Engineering Inc., Stamford, Ct., U.S.A.), and cooled at a rate of -0.5^{0}C/min. to terminal temperatures of 0 (control), -15, -30, -45 and -60^{0}C. At the designated terminal cooling temperature, samples were removed to be immediately thawed, or immersed in liquid nitrogen (-196^{0}C) for 30 minutes before thawing. Samples were thawed just until the frozen pellet had melted, in a 40^{0}C water bath, and placed on ice until the remaining samples had been treated and were ready for transfer (up to 2 hours). The samples were pipetted under sterile conditions into (100x 15mm) petri dishes containing 9.4 mL of liquid culture media at a final concentration of approximately 6x 10^4 microspores/mL.

Post-treatment Culture

Petri plates containing cryo-treated microspores were sealed with parafilm and placed in an incubator at 30^{0}C in the dark for 14 days. The plates were placed on a rotary shaker (60 rpm) at room temperature in the dark. At 30 days, the numbers of cotyledonary embryos were recorded and the embryos transfered to (100x 15mm) petri plates containing B5 solid media supplemented with 0.15mg/L of gibberellic acid (Appendix C). The plates, each containing 20 embryos, were incubated for 10 days at 4^{0}C with an 8 hour photoperiod under a photon flux density of 30 μmol $m^{-2}s^{-1}$. Subsequently, the plates were incubated at room temperature (24^{0}C), with a 12 hour photoperiod under a photon flux density of 30 μmol $m^{-2}s^{-1}$, until the embryos germinated and formed true

leaves (30-40 days). The numbers of surviving plants per treatment were recorded.

Plantlet Transfer and Plant Regeneration

Plantlets (germinated embryos) were transferred to multiwell transfer flats containing moist soil-free mix (metro mix). Plantlets were watered, misted and covered with clear plastic to maintain humidity. After one week the plastic was removed and the plantlets watered regularly with a solution containing 1 g/L of (20-20-20 [N:P:K]) fertilizer.

Ploidy Determination

Plant ploidy level was determined by flower morphology (Keller and Armstrong 1978). Haploids produced anthers lacking pollen, and flower buds which atrophied. Diploid plants had anthers which produced an abundance of pollen and had good seed set.

As a comparison, flow cytometric analysis was used to determine ploidy levels in single embryo samples derived from the control, and microspores cooled to -45^{0}C with and without subsequent immersion in liquid nitrogen. Nuclei were isolated according to the methods of Ulrich and Ulrich (1991). Embryos (2 g) were minced with a sharp razor blade in 2 mL of chopping buffer containing 0.1M citric acid and 0.5% (v/v) tween 20, incubated for 20-30 minutes at 0^{0}C, filtered through 40 μm nylon mesh and fixed in 3:1 (ethanol: acetic acid). Isolated nuclei were then centrifuged at 200x g for 15 minutes and resuspended in 1.0 mL staining solution (pH 7.0) containing 0.4M sodium hydrogen phosphate and 100 μg/mL propidium iodide. RNAase (2 mg/mL) in 0.4 sodium hydrogen

phosphate solution was added (1.0 mL) to the stain solution and the contents allowed to incubate for 30 minutes at 37°C. The nuclei were then filtered through 40 µm nylon mesh and flow cytometric analysis carried out with a FACScan flow cytometer (Becton Dickinson Immunocytometry, Mountain View, USA). Histograms of DNA nuclear content were compared with results from ploidy analysis of mature plants.

Statistical Analysis

The experiment was repeated twice, each with two replicates of 20 transferred embryos per treatment; a total of 80 embryos per treatment were transferred. The means of the various treatments were compared with Duncan's multiple range test (Steel and Torrie 1980).

3.3.4 Results

Plant Population Regenerated

A total of 176 plants were produced from all of the treatments. Of these 70 (39.8%) were haploid and 106 (60.2%) were diploid. No mixoploid or tetraploids were observed in the plants regenerated.

Effect of Cryo-treatment on Embryo Yield

The comparison of treatment means (Duncan's multiple range test) (Table 3.3.1), shows that non-immersed samples cooled to -30 and -60°C had embryo yields which were not significantly different from the control. Samples cooled to -15 and -45° C had lower

embryo yields which were significantly different than the control. The comparison of means also showed that samples treated with liquid nitrogen resulted in embryo yields that were significantly different from either the control or non-immersed samples.

The number of embryos produced was calculated as an average from four replicates per treatment. Each replicate (petri dish) had approximately 6x 10^5 microspores per 10 mL of culture medium. Yields were highest in the control at 2244 embryos/plate (100%). In cooled samples, that were not immersed in liquid nitrogen, embryo yields ranged from 60.0 to 89.3% and there was no definite relationship between terminal temperature and embryo yield (Figure 3.3.1). Liquid nitrogen immersion was lethal unless microspore samples were cooled to at least -45^{0}C. With terminal temperatures of -45 and -60^{0}C liquid nitrogen immersion resulted in embryo yields of 37.0 and 31.7% that of the control value respectively. Although nitrogen immersed samples had lower embryo yields, the embryos produced were more uniform and fewer proembryo-like structures were present (Figure 3.3.5).

Effect of Cryo-treatment on Plant Regeneration

The comparison of treatment means (Duncan's multiple range test) (Table 3.3.1), shows that the -45^{0}C sample immersed in liquid nitrogen produced significantly better plant regeneration. The -60^{0}C sample also produced high plant regeneration but was not significantly different from either the -45 C immersed sample or the control. Samples which were not immersed in liquid nitrogen produced low regeneration frequencies and were not significantly different from the control.

The percentage of plants regenerated was measured from 20 transferred embryos per replicate or, as a total, out of 80 per treatment. Control samples produced an average regeneration frequency of 30.0% (Figure 3.3.2). Cooled samples which were not immersed in liquid nitrogen had regeneration frequencies ranging from 15.0% to 27.5% with regeneration decreasing as terminal temperature decreased. Samples immersed in liquid nitrogen produced better regeneration frequencies than non-immersed counterparts; 61.3% and 43.8% regeneration for -45 and -60^{0}C samples respectively. These samples initially also had the lowest embryos yields.

Effect of Cryo-treatment on Diploidization Frequency

A comparison of diploidization frequency means (Duncan's multiple range test) (Table 3.3.1), showed that diploidization increased as the terminal temperature decreased, irrespective of whether the samples were immersed in liquid nitrogen. The means of samples immersed in liquid nitrogen, however, were not significantly different from their non-immersed counterparts.

A clear and direct relationship appears to exist between the terminal temperature reached by cooled microspore samples and the frequency of diploid plants regenerated following microspore culture. Diploidization frequency increased as terminal temperature decreased from 22.5% in the control to 87.5% at -60^{0}C (Figure 3.3.3). Samples immersed in liquid nitrogen were comparable to non-immersed samples with frequencies of 66.7% and 79.9% for -45 and -60^{0}C samples respectively. Liquid nitrogen immersion did not affect the ploidy level.

Flow cytometric analysis indicated a major shift in ploidy level of microspore-derived embryos due to decreasing terminal temperature (Figure 3.3.6). Control cultures (Figure 3.3.5 a) produced a population of embryos which were mainly haploid, as indicated by the presence of a large 1C peak (G0/G1 phases of the cell cycle) and a smaller 2C peak (G2 and mitotic phases of the cell cycle)(Figure 3.3.6 a). The cooling of microspores to a terminal temperature of -45^{0}C caused a major shift from haploid to diploid ploidy level of microspore-derived embryos (Figure 3.3.5 b,c), as evidenced by the presence of a larger 2C peak and a new 4C peak (Figure 3.3.6 b,c). The haploid peaks (1C and 2C) overlap the diploid peaks (2C and 4C) in the -45^{0}C samples.

3.3.5 Discussion

The induction of sporophytic development and chromosome doubling in *B. napus* microspores is dependent on events which occur during the first 24 hours of culture. Physical and chemical stresses applied to microspores during this period, directly affect both the initial symmetry and completion of cell division. Depending on the strength and duration of the stress involved both embryogenesis and/or chromosome doubling frequencies are increased. Treatment of microspores with low temperature, anti-microtubule agents, and cryo-treatment increase the percentage of microspores which undergo sporophytic development and/or chromosome doubling (Charne et al. 1988; Eikenberry 1994; Iqbal et al. 1994; Lo and Pauls 1992; Möllers et al. 1994).

It was reported more than 50 years ago that decreased temperature and/or temperature shocks can interfere with the developmental pathway of pollen (Sax 1937).

Temperature variation can result in the production of unreduced gametes and/or prevent the normal asymmetric division during pollen mitosis I from occurring. The significance of such a change in division symmetry, and its subsequent influence on embryogenesis of *B. napus* microspores *in vitro*, has only recently been determined (Zaki and Dickinson 1991). This is most likely the reason why microspore donor plants of *B. napus* grown at lowered temperature produce microspores which are more embryogenic than ones grown at higher temperature (Lo and Pauls 1992).

Decreased temperature is known to prevent the proper polymerization of microtubules and formation of microtubule networks (Carter and Wick 1984). The process of nuclear positioning during pollen mitosis I (late uninucleate stage of microspores) presumably requires that the cytoskeleton and microtubule network be properly functioning. Microtubules and actin microfilaments, along with the large microspore vacuole, are responsible for both positioning of the nucleus and cell plate (Morejohn 1991; Hause et al. 1992). Under normal conditions, microtubules participate in the asymmetric division which preceeds gametophytic development of microspores. When microspores are subjected to decreased temperature asymmetric positioning and cell division can be inhibited, the position of the nucleus is disrupted, and it is positioned in the center of the microspore cytoplasm. When placed in culture, these microspores divide symmetrically to produce two cells of roughly equal size and, subsequently, sporophytic development may occur (Zaki and Dickinson 1991). As with low temperatures, stresses incurred during cryo-preservation can damage microtubules. Subjecting plant cells to freezing temperatures can cause not only depolymerization of microtubules but also

inhibited recovery of microtubule networks after treatment (Bartolo and Carter 1991). Freezing may cause damage to membrane systems of cells that have secondary effects on microtubules. Damage to the endoplasmic reticulum and mitochondria can result in the release of calcium into the cytoplasm (Ferguson et al. 1985; Monroy et al. 1993). At mM concentrations calcium causes the depolymerization of microtubules and thus may inhibit processes which require functioning microtubules (Hepler and Wayne 1985). Freeze-induced dehydration may also cause preciptation of proteins, including tubulin (Tamiya et al. 1985).

Cells that are cryopreserved are affected by different stresses during cooling and thawing (Mazur 1969, 1970). If ice forms within the cytoplasm lethal damage to membranes can occur. Slow cooling enables supercooled water in the cytoplasm to move out into the extracellular space where it forms ice (Steponkus 1984). Although this reduces ice damage, excessive dehydration can occur. The plasma membrane has to reduce its volume during dehydration of the cytoplasm and again increase its volume during thawing as water is reabsorbed (Dowgert and Steponkus 1984). Inability to lose and reincorporate membrane material can cause the membrane to fracture or lyse. Similarly, membranes can undergo phase changes, lamellar to hexagonal II transition, when there is excessive dehydration (Gordon-Kamm and Steponkus 1984a; Grout and Morris 1987). Excessive dehydration and concentration of cytoplasmic constituents can cause proteins to lose their quaternary and tertiary structure resulting in their precipitation (Tamiya et al. 1985). Changes in pH, electro-chemical and other characteristics of the cytoplasm may also result in damage (Arakawa and Timasheff 1985; Carpenter and

Crowe 1988).

To protect cells from potential sources of damage during freezing, there are a number of treatments and techniques which can be applied. These include the use of vigorous starting material, cold acclimation, cryoprotectants, proper cooling regime, careful thawing, and post-treatment of cryopreserved material.

Plant cells that have been previously exposed to decreased temperatures are often better equipped to withstand the effects of freezing. Alteration in gene expression, proteins, membrane composition, and biochemical pathways, induce a number of physiological changes which allow cells to better withstand the effects of low temperature and freezing (Gordon-Kamm and Steponkus 1984b; Guy 1990). Low temperatures, dessication stress, and ABA, have been shown to induce a number of physiological and biochemical alterations which increase freezing tolerance in *B. napus* (Harada et al. 1989; Orr et al. 1990; Johnson-Flanagan et al. 1991). Donor plants grown at low temperature produce a population of microspores which is more embryogenic (Lo and Pauls 1992). Microspore donor plants in this experiment were grown at low temperatures (10/5^{0}C day/night) in contrast to that of other researchers (23/19^{0}C day/night) (Charne et al. 1988; Chen and Beversdorf 1992b). Although survival or embryogenesis was much better in this experiment than observed previously in cryotreatments with the same cultivar, it is possible that low temperatures induced cold acclimation which, in turn, led to increased survival. By using microspores from donor plants grown at reduced temperature, a population of microspores is produced which is more embryogenic and in better physiological condition to withstand damage from cryopreservation.

Cryoprotectants protect cells from damage during freezing by a number of mechanisms. These include depressing the freezing point of the cytoplasm, preventing dehydration, and stabilization of proteins and membranes, and by other mechanisms (Anchordoguy et al. 1987; Carpenter and Crowe 1988). In this experiment, the only cryoprotectant used was NLN culture medium containing 13% sucrose. Sucrose occurs as a natural cryoprotectant in plant cells and its levels usually increase in cells which are subjected to cold hardening conditions. It has been shown that sucrose can prevent dehydration of proteins and membranes by excluding their interaction with the other components of the cytoplasm (Anchordoguy et al. 1987; Lee and Timasheff 1981; Strauss and Hauser 1986). Sucrose increases the concentration of the cytoplasm which prevents water loss, and further depresses its freezing point. Unlike many artificial cryoprotectants, sucrose is relatively non-toxic. Previous experiments in which *B. napus* microspores were cryopreserved have also used NLN containing 13% sucrose as a cryoprotectant (Charne et al. 1988; Chen and Beversdorf 1992a,b).

Both the cooling rate and terminal temperature have distinct effects on cell hydration. The cooling rate and terminal temperature have to be properly determined to prevent damage from ice formation or from excessive dehydration. As cells are cooled, water is removed from the cytoplasm to the extracellular space where it forms ice. Removal of water from the cytoplasm is directly related to the terminal temperature to which cells are cooled (Grout and Morris 1987). If the cooling rate is too fast there is little time for water to be removed from the cytoplasm. This results in the formation of ice crystals within the cytoplasm and irreversible damage to membrane systems.

Alternately, if the rate of cooling is slow then excessive dehydration of the cytoplasm can occur.

In this experiment a cooling rate of -0.5^{0}C/min. was used. Charne et al. (1988) had used a cooling rate of -0.25^{0}C/min. and Chen and Beversdorf (1992b) similarly found that a cooling rate of -0.25^{0}C/min. produced better results than cooling rates of -1.0 or -0.03^{0}C/min. The rate chosen in this experiment was chosen for convenience. Steps of the cryopreservation procedure, from isolation of microspores to storage or final plating of thawed microspores, could be completed within approximately four hours. A cooling rate of -0.25^{0}C/min. would require more than four hours for the cooling procedure alone.

Previous experiments in which microspores were cryopreserved have utilized microspores isolated from donor plants grown with a 23/19^{0}C day/night temperature regime (Charne et al. 1988; Chen and Beversdorf 1992a,b). In the experiment of Charne et al. (1988), using *B. napus* cv. Topas, the controls produced an average of 57.2 embryos per 2x 10^5 plated microspores. Microspores which were cooled to -15^{0}C and plunged into liquid nitrogen produced an average of only 0.46 embryos per 2x 10^5 plated microspores. Both the control and nitrogen treated material of Charne et al. (1988) produced embryo yields far lower (7.6% or less) than observed in this experiment. Although the same genotype was used in this experiment, donor plants were grown at low temperatures (10/5^{0}C day/night) and cryopreservation in liquid nitrogen was not successful until terminal temperatures of -45 and -60^{0}C were reached. In the experiment of Chen and Beversdorf (1992b) the genotypes G-231 and M3-124 were tested. A cooling rate of -0.25^{0}C/min. and terminal temperature of -35^{0}C were found to produce the best results for

microspores subsequently immersed in liquid nitrogen. G-231 and M3-124 cryopreserved microspores produced embryo yields that were 18.0% and 40.0% of their respective control values of 260 and 3443 embryos per 8x 10^5 plated microspores.

In the present experiment samples cooled to -60^{0}C, without cryopreservation, produced embryo yields comparable to the control (Figure 3.3.1). Since water is removed from cells in a strictly temperature-dependant manner, dehydration damage should increase with decreasing terminal temperature. Therefore, the non-immersed -15 and -45^{0}C samples should also have had similar embryo yields. The relatively high yields of the -60^{0}C sample suggests that the cooling rate was adequate for water to move from the cytoplasm to the extracellular space at a rate sufficient to prevent damage from ice formation or from excessive dehydration. Liquid nitrogen immersion proved to be lethal for samples that had not been cooled to a sufficiently low terminal temperature. The lethal damage that occurs is most likely due to excessive water remaining in the cytoplasm and the formation of ice as samples are placed in liquid nitrogen. The cytoplasm can supercool to below -40^{0}C if the rate of cooling is slow enough for water removal to the extracellular space to occur (Rasmussen et al. 1975). As the cytoplasm is dehydrated the increased solute concentration further depresses the freezing point and prevents ice formation. Samples which were cooled to -45 and -60^{0}C, with liquid nitrogen immersion, produced approximately one third the number of embryos produced by the control. Damage may have occurred during the process of immersion itself or during thawing when ice re-crystallization and related stress damage can occur.

Samples which were cooled to -45 and -60^{0}C with liquid nitrogen immersion,

produced higher plant regeneration frequencies than the other treatments (Figure 3.3.2). These samples also had a lower embryo yield. Because of the lower number of embryos, nutritive conditions may have favored the development of embryos of better quality. Consequently, this may have resulted in improved regeneration frequencies. Since the microspores tested were all of one genotype, selection of microspores in different stages of development or physiological condition may have occurred. Microspores and/or pollen grains which do not undergo embryogenesis may persist and produce toxic compounds that inhibit embryo development (Kott et al. 1988). Elimination of this non-embryogenic material may prevent the occurence of such compounds. No tests were conducted to determine if this situation occurred.

The effect of cryopreservation on the frequency of diploidization of microspore-derived *B. napus* was originally demonstrated by (Charne et. al. 1988). Despite limited success, a diploidization frequency of 88.0% was obtained from microspore samples cooled to -15^{0}C followed by liquid nitrogen immersion. Chen and Beversdorf (1992a) later demonstrated that as the terminal cooling temperature decreased the frequency of chromosome doubling increased. Liquid nitrogen immersion was also shown to have no effect on diploidization. These researchers obtained maximal doubling frequencies of 51.1% and 47.7% in the genotypes G-231 and M3-124 after cooling to a terminal temperature of -40^{0}C. The same effects were also observed in this experiment as well (Figure 3.3.3). The primary change that occurs during cooling to lower terminal temperatures is increased dehydration as supercooled liquid water moves from the cytoplasm to the extracellular space. If these researchers had used lower terminal

temperatures to increase the amount of dehydration, as in this experiment, doubling frequencies may have even been higher.

The origins of cryo-induced doubled haploids are still unknown. It has been suggested that selection of spontaneously doubled microspores or unreduced microspores occurs *in vitro* (Chen and Beversdorf 1992a), however, this is most likely not the case. In the present study the control produced 22.5% diploids. Compared to the -45 and -60°C liquid nitrogen immersed samples, where the survival rate was approximately 1/3 that of the control and the doubling frequency in excess of 80%, the idea that doubled microspores can survive preferentially to haploid microspores would be reasonble. However, compared to the -45 and -60 non-immersed samples, where the doubling frequency was in excess of 80% but the survival rate was 60 and 80% respectively, selection of unreduced microspores does not appear to occur. Many of the microspores in these samples were clearly affected by cryo-induced changes which subsequently led to chromosome doubling. Unreduced gametes and microspores of increased ploidy tend to be larger in size. Because of the smaller ratio between plasma membrane surface area and cytoplasmic volume, these microspores lose water more slowly than normal reduced microspores. This phenomenon should predispose microspores of larger size and higher ploidy to be more susceptible to cryo-damage effects, and selection against their survival should actually occur. Changes in calcium concentrations, precipitation of tubulin subunits and other events that affect recovery of microtubule networks after treatment have not been investigated. Possibly, such changes aid the process of cryo-induced chromosome doubling in microspores of *B. napus*.

Table 3.3.1 Mean percentage values for embryo yield, plant regeneration and diploidization following cryo-treatment of *B. napus* microspores.

Terminal Temperature	N_l (- 196 °C) Immersion	% Embryo Yield	% Plant Regeneration	% Diploids
0 °C	-	100.0 a	30.0 bc	22.5 c
	+	0		
-15 °C	-	64.0 b	22.5 cb	39.5 bc
	+	0		
-30 °C	-	89.3 a	27.5 bc	44.4 bc
	+	0		
-45 °C	-	60.0 b	15.0 c	63.8 ab
	+	37.0 c	61.3 a	66.7 ab
-60 °C	-	84.9 a	15.0 c	87.5 a
	+	31.7 c	43.8 ab	80.0 a

Note: the control (@ 0°C, - nitrogen immersion) produced an average of 2244.0 (± 226.9) embryos per 6 x 10^5 plated microspores. Plant regeneration and diploidization values are out of four replicates, each replicate composed of 20 transferred embryos.

Values which are followed by the same letter(s) are not significantly different according to Duncan's multiple range test.

The pooled standard error values were (± 113.7) embryos per 6 x 10^5 plated microspores or ±5.1% (embryo yield), ±7.1% (plant yield) and ±9.1% (diploidization).

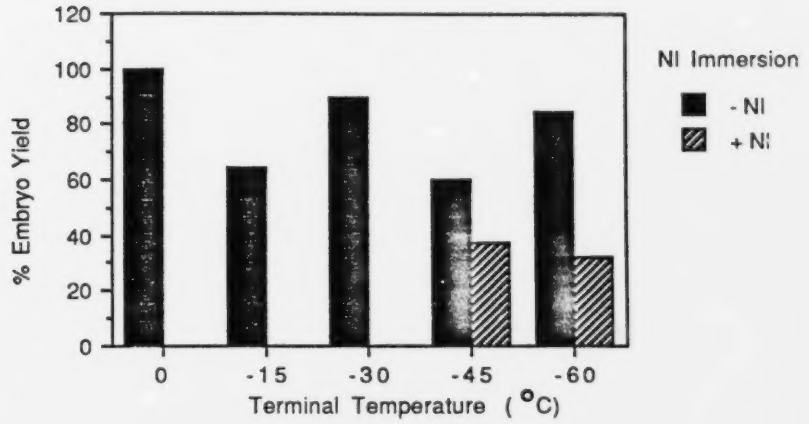

Figure 3.3.1 Effect of terminal temperature and (±) liquid nitrogen immersion of *B. napus* microspores on subsequent embryo yield. (Pooled SE= ±5.1%).

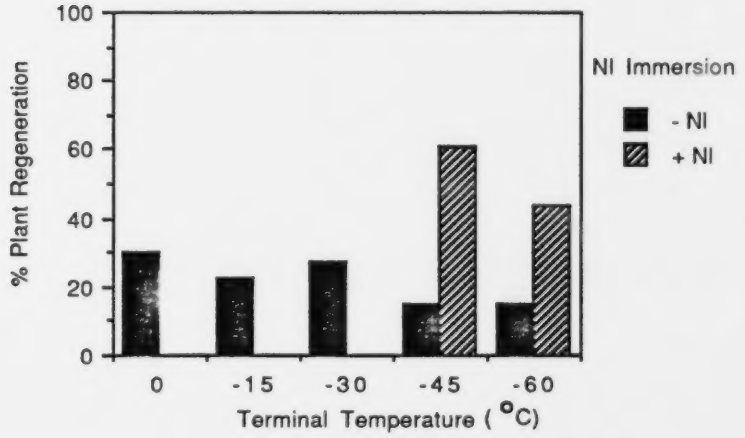

Figure 3.3.2 Effect of terminal temperature and (±) liquid nitrogen immersion of *B. napus* microspores on subsequent plant regeneration. (Pooled SE= ±7.1%).

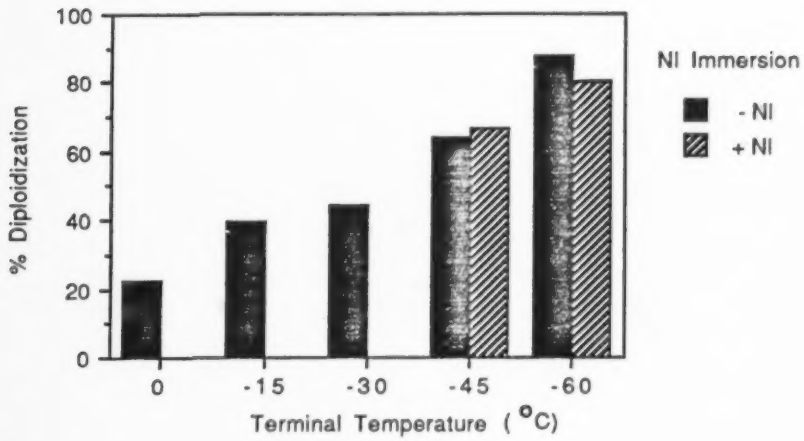

Figure 3.3.3 Effect of terminal temperature and (±) liquid nitrogen immersion of *B. napus* microspores on subsequent diploidization. (Pooled SE= ±9.1%).

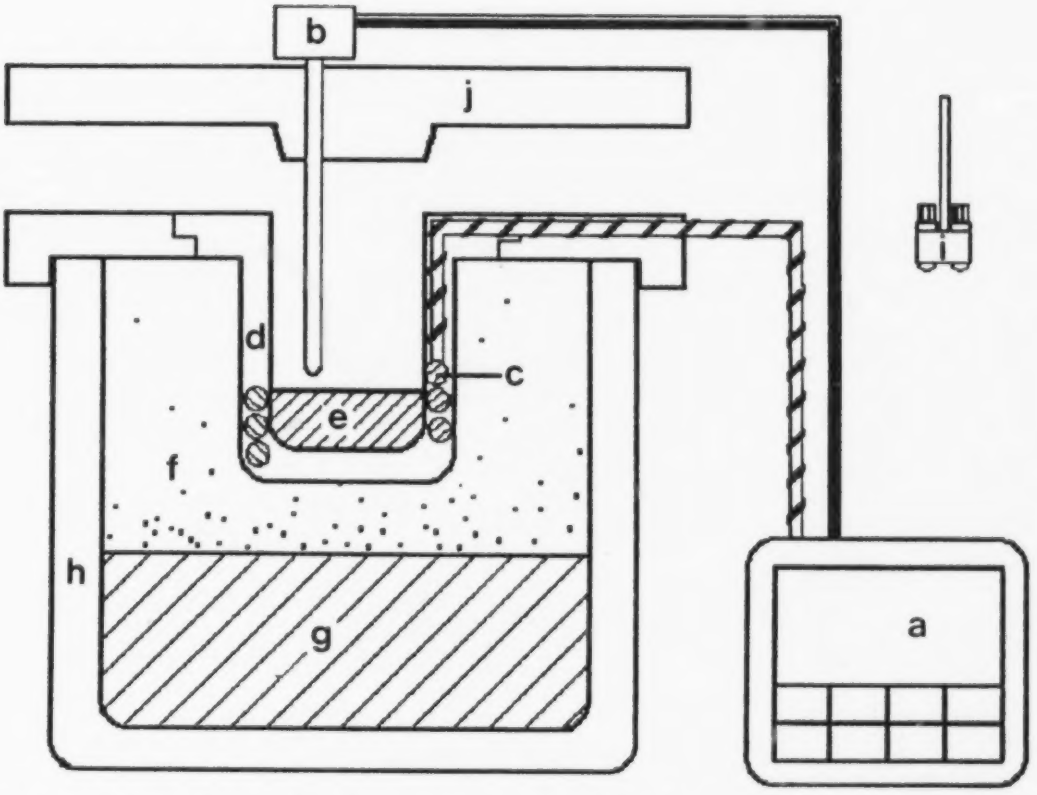

Figure 3.3.4 Schematic diagram of cryo-cooler. The cooling unit consists of a programmable temperature controller (**a**), Omega series CN-2010 (Omega Engineering Inc., Stamford, Ct., U.S.A.), connected to a temperature probe (**b**) and heat tape (**c**) surrounding the cooling chamber (**d**). Alcohol in the cooling chamber (**e**) is cooled by nitrogen vapour (**f**) produced by liquid nitrogen (**g**) in the underlying thermos flask (**h**). Specimens to be cooled are placed in cryovials (**i**), taped to plastic straws, placed into the cooling chamber and covered with a styrofoam lid (**j**).

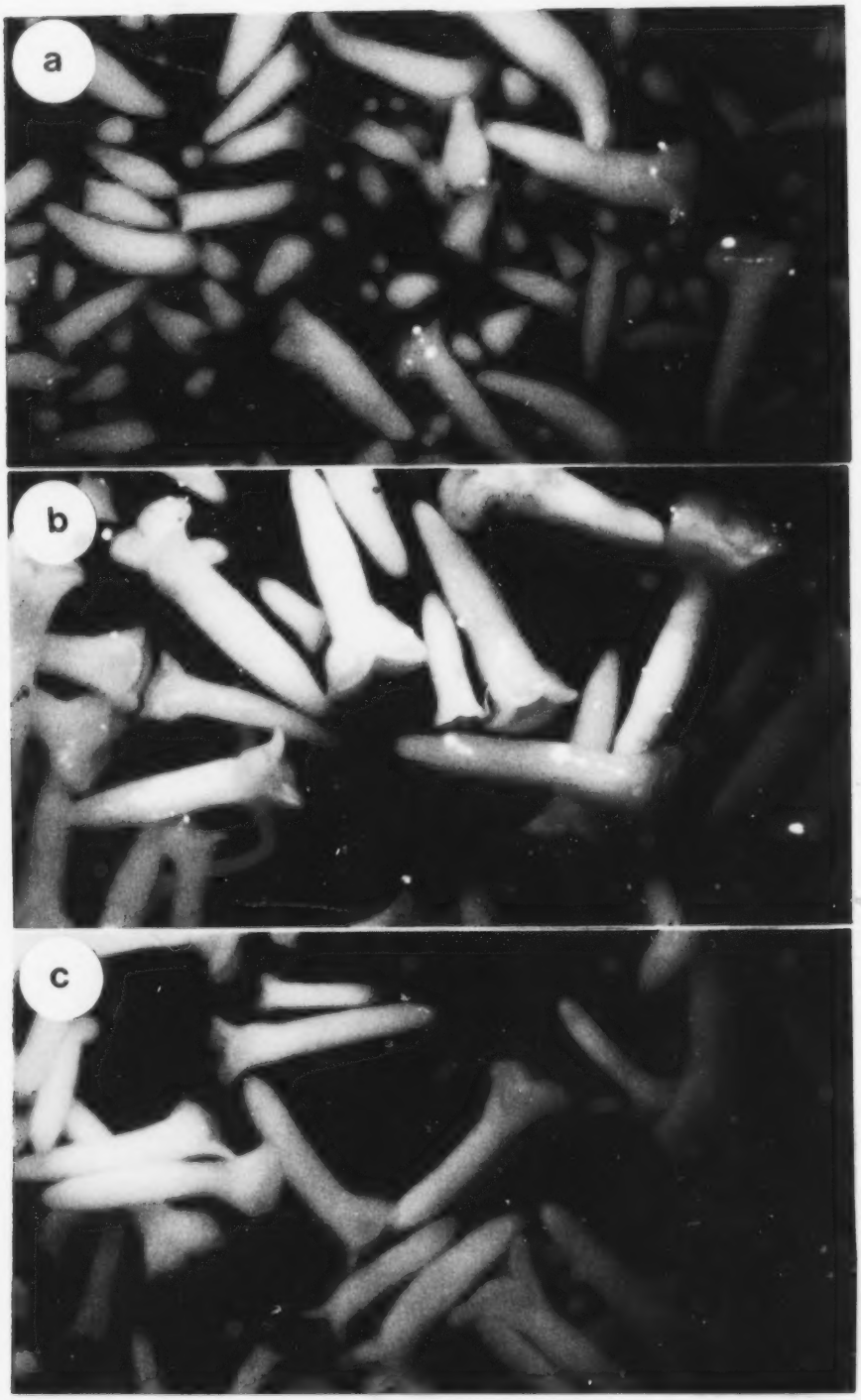

Figure 3.3.5 Appearance of microspore-derived *B. napus* embryos corresponding to the histograms in Figure 3.3.6; a) control, b) cooled to -45^{0}C, and c) cooled to -45^{0}C with subsequent immersion in liquid nitrogen.

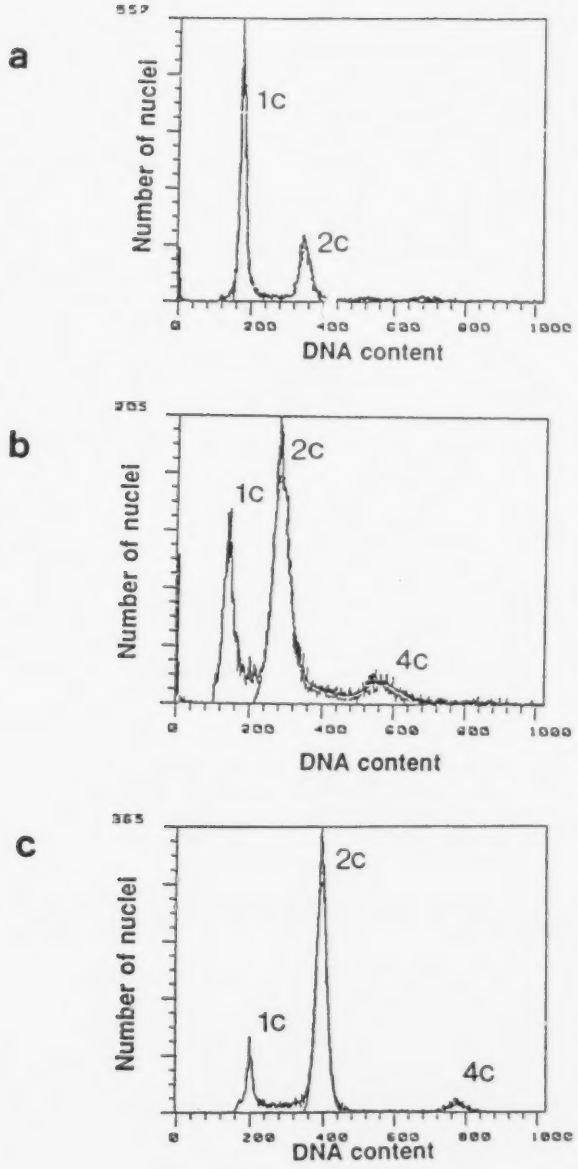

Figure 3.3.6 DNA histograms from flow cytometric analysis of isolated nuclei showing DNA peaks from *B. napus* embryos derived from a) control cultures, b) from microspores cooled to -45, and c) from microspores cooled to -45°C with subsequent immersion in liquid nitrogen.

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3.4 Experiment #4: Cryo-induced Chromosome Doubling of Microspores in *Brassica napus* L. cv. Topas: Effect of ice (0°C) pre-treatment and sucrose.

3.4.1 Abstract

The influence of ice pre-treatment, sucrose concentration, and cooling regime on subsequent microspore embryogenesis and diploidization frequency in *Brassica napus* L. cv. Topas following cryo-treatment was tested. Microspore samples placed in cryoprotectant media consisting of 13, 25 and 50% sucrose were cooled at 0.5°C/min. to terminal temperatures of -15, -30, -45 and -60°C followed by immediate thawing or immersion in liquid nitrogen. Ice pre-treatment of microspore donor buds significantly increased the ability of microspores to withstand the effects of liquid nitrogen storage. Nearly all of the samples that were immersed in liquid nitrogen, following cooling to a given terminal temperature, produced embryogenic frequencies comparable to their non-immersed counterparts. Although a cryoprotectant medium containing 25% sucrose improved survival at the lower ranges of terminal cooling temperature, overall embryo production tended to decreased with increased sucrose concentration and with decreasing terminal temperature. Diploidization frequency appeared to be related to pre-disposing conditions induced by ice-pretreatment of buds and events occuring during the period of cooling between 0 and -15°C. Microspore samples placed in cryoprotectant solution containing 13% sucrose and cooled to -15°C at 0.5°C/min., produced the highest embryogenic frequencies (embryo yields) of 71.8 and 77.3% that of the control for non-immersed and liquid nitrogen immersed samples respectively. Relative to the control value of 29.3%, diploidization frequencies for samples cooled to -15°C or lower were uniformly

high, ranging from 63.7 to 93.7%. This experiment illustrates the usefulness of applying low temperature pre-treatment for improved survival and diploidization of *B. napus* microspores following cryopreservation.

3.4.2 Introduction

By applying microspore culture in a *B. napus* breeding program, homozygous lines can be produced within a single generation. This method is far more effective than conventional breeding techniques which can require ten or more years to produce and evaluate a homozygous inbred line. In recent years, there have been many improvements in treatment of microspore donor plants and culture conditions which have led to increased embryo yields and application of microspore culture technique to a wider range of germplasm. However, the potential exists to develop and improve methods used to double the chromosome number of microspore-derived plants. Cryo-treatment of microspores has been shown to increase the frequency of spontaneous diploidization of subsequent microspore-derived plants (Charne et al. 1988; Chen and Beversdorf 1992a). Therefore, the requirement for inducing chromosome doubling at early flowering stages can be decreased. Development of improved protocols for cryopreservation of *B. napus* microspores would result in better utilization of germplasm, space, and financial resources in a breeding program.

To protect cells from damage during cryopreservation it is important that care is taken to pre-condition material and/or use a cryoprotectant medium and cooling regime that ensures an adequate level of survival. Cold acclimation has been shown to induce

physiological changes in plant cells which provide resistance to damage incurred at low temperatures and during freezing (Guy 1990). Similarly, pre-treatment of cells with low temperature may encourage increased survival during cryopreservation. In previous research work on cryopreservation of microspores, the effects of pre-conditioning microspores with low temperature and alteration of cryoprotectant osmotic conditions have not been investigated. The objectives of this experiment were to study the effect of ice pre-treatment, sucrose concentration in the cryoprotectant medium, and cooling regime, on subsequent embryogenesis and chromosome doubling of *B. napus* L. cv. Topas microspores following cryo-treatment.

3.4.3 Materials and Methods

Growth of Microspore Donor Plants

Procedures followed for growing donor plants and microspore culture were those of Coventry et al. (1988) and Swanson (1990). Seeds of *B. napus* L. cv. Topas were germinated at 18^{0}C on moist filter paper in petri dishes. Seedlings were transferred to 6-inch pots containing metro mix (W.R. Grace & Co. Canada) soil-free growth media. The pots were placed in a growth cabinet maintained at 24/21^{0}C day/night temperature with a 16 hour photoperiod and photon flux density of 425-450 µmol m^{-2} s^{-1}. After reaching the four-leaf stage the number of plants per pot were reduced to one. Plants were watered as required, 3 to 4 times weekly, with a solution containing 1 g/L of (20-20-20 [N:P:K]) fertilizer. As the first buds began to form, the temperature in the growth chamber was reduced to 10/5^{0}C day/night.

Ice Pre-treatment

Racemes with buds at the proper stage of development (1.5-3.5 mm in length) were removed from donor plants, wrapped in moist paper towel, placed on a layer of ice, covered loosely with chopped ice and placed in a fridge in the dark at 0-1^{0}C for 24 hours.

Microspore Isolation

Buds were surface sterilized in a solution of sodium hypochlorite 7.0% (w/v) for 10 minutes and rinsed three times for 5 minutes with sterile ice cold water. The buds were gently crushed with a pestle and mortar in B5 isolation medium containing 13% (w/v) sucrose (Appendix A) (Lichter 1982), and the homogenate filtered through a 63 and 44 μm nylon mesh (Nytex). The filtrate was then poured into a 50 mL Falcon tube (Fisher Scientific, Canada) and centrifuged at 250g for 10 minutes. The microspore pellet was then washed and centrifuged two more times with B5 medium. The microspores were then resuspended in modified NLN medium (Appendix B) (Lichter 1982), containing 13, 25 or 50% (w/v) sucrose. The microspore concentration was adjusted, using a haemocytometer, to approximately 1x 10^6 microspores/mL. Samples (0.6 mL) were placed into 1 mL cryovials and allowed to equilibrate on ice (0^{0}C) for 2 hours.

Cryo-treatment

Microspore samples were placed in a programmable cryo-cooler and cooled at a rate of -0.5^{0}C/min. to terminal temperatures of 0, -15, -30, -45 and -60^{0}C. Upon reaching the designated terminal cooling temperature samples from each sucrose concentration

were removed and immediately thawed or immersed in liquid nitrogen (-196^{0}C) for 30 minutes before thawing. Samples were thawed carefully in a 40^{0}C water bath and placed on ice just after the frozen pellet had melted. Cryovials were swabbed with 70% ethanol before opening to transfer microspores. The samples were pipetted under sterile conditions into (100x 15mm) petri dishes containing 9.4 mL of liquid culture media to produce a final concentration of approximately 6x 10^4 microspores/mL.

Post-treatment Culture

Petri plates containing cryo-treated microspores were sealed with parafilm and placed in an incubator at 30^{0}C in the dark. After 14 days the plates were placed on a rotary shaker (60 rpm) at room temperature in the dark. At 30 days of development the numbers of cotyledonary embryos were recorded and the embryos were transfered to petri plates (100x 15mm) containing B5 solid media supplemented with 0.15 mg/L of gibberellic acid (Appendix C). The plates (containing 20 embryos) were first incubated for 10 days at 4^{0}C with an 8 hour photoperiod under a photon flux density of 30 µmol m^{-2} s^{-1}. Subsequently, the plates were incubated at room temperature (24^{0}C), with a 12 hour photoperiod under a photoflux density of 30 µmol m^{-2} s^{-1}, until the embryos germinated and formed true leaves (30-40 days). The numbers of surviving plants per treatment were recorded.

Plantlet Transfer and Plant Regeneration

Plantlets (germinated embryos) were transferred to multiwell transfer flats

containing moist soil-free mix (metro mix). Plantlets were watered, misted and covered with clear plastic to maintain humidity. After one week the plastic was removed and the plantlets watered regularly with a solution containing 1 g/L of (20-20-20 [N:P:K]) fertilizer.

Ploidy Determination

Plant ploidy level was determined by counting the chloroplast number in guard cells of expanded leaves (Chevre et al. 1989; Lucas et al. 1991). Two leaf disks per plant were removed from the abaxial side of leaves and placed on a glass slide with the abaxial side facing upwards. A few drops of 1.0% (w/v) silver nitrate solution were placed on each for 3 to 5 minutes. A glass coverslip was then placed over the disks, and guard cell chloroplasts in stomates around the outer edge of the leaf disks were counted under the magnification of a standard light microscope.

Statistical Analysis

The experiment was repeated twice, each with two replicates of 20 transferred embryos per treatment; a total of 80 embryos per treatment were transferred. The means of the various treatments were compared with Duncan's multiple range test (Steel and Torrie 1980).

3.4.4 Results

Plant Population Regenerated

A total of 618 plants were regenerated from all of the treatments. Of these, 158 (25.6%) were haploid, 452 (73.1%) diploid and 8 (1.3%) tetraploid. No mixoploid plants were produced from any of the treatments. The 8 tetraploids plants produced may have been derived from unreduced microspores or spontaneous diploids which had undergone doubling. The low percentage of tetraploids (1.3%) relative to the rest of the population, and the fact that seed set was low suggests they have little influence on the efficiency of chromosome doubling.

Effect of Cryotreatment on Embryogenesis

The comparison of treatment means (Duncan's multiple range test) (Table 3.4.1), showed that the control mean embryo yield was significantly different from the other treatments with the remainder of the treatment means showing significant decreases as the terminal temperature decreased and as sucrose concentration increased. However, the 25% sucrose cryoprotectant solution produced significantly better survival rates at lower terminal cooling temperatures compared to the other treatments. Embryo yields were highest in the control with an average of 2790 embryos per plate (100%). For treated samples, maximal values for survival were obtained from samples placed in 13% sucrose and cooled to -15^{0}C; at 71.8% for non-immersed and 77.3% for nitrogen immersed samples. The lowest embryo yields were produced in samples placed in 50% sucrose and cooled to -60^{0}C without immersion (30.0%) and with liquid nitrogen immersion (30.7%).

Compared to previous experiments in which there was no ice pre-treatment of microspore donor buds, the ice pre-treatment appeared to provide better protection from the effects of liquid nitrogen immmersion. Overall, there was little difference between samples with or without subsequent liquid nitrogen immersion after cooling.

Effect of Cryotreatment on Plant Regeneration

From the comparison of treatment means (Duncan's multiple range test) (Table 3.4.1), significant variation between individual means exists. However, there did not appear to be any obvious relationship between type of treatment and plant regeneration frequency; as seen in (Figure 3.4.2, 3.4.5, 3.4.8). Plant regeneration frequencies ranged from 12.5 to 50.0%. The control produced an average regeneration frequency of 38.8%.

Effect of Cryotreatment on Diploidization

The comparison of individual treatment means (Duncan's multiple range test) (Table 3.4.1), showed that the average diploidization of the control was significantly different from the rest of the treatment means. The individual treatment means showed virtually no variation from one another. The control produced the lowest overall average diploidization frequency at 29.3%. The highest diploidization frequency, 93.7%, was produced from samples placed in 25% sucrose followed by cooling to -60^{0}C with subsequent immersion in liquid nitrogen. The diploidization frequencies for most treatments tended to be between 70.0% and 90.0%.

3.4.5 Discussion

B. napus germplasm has been cryopreserved in the form of shoot tips, embryos, cell suspensions and microspores (Weber et al. 1982; Charne et al. 1988; Withers et al 1988; Uragami et al. 1993; Chen and Beversdorf 1992a,b). Most of these techniques have involved preculturing of plant material in cryoprotectant media followed by direct immersion in liquid nitrogen (vitrification), or slow cooling to approximately -40 ^{0}C before immersion in liquid nitrogen. Survival rates in excess of 50% have been reported for most protocols, with some protocols resulting in an excess of 90% survival. However, difficulties often exist in regeneration of plants from such treatments. Problems with removal of cryoprotectants and excessive callusing of tissue can lead to inhibition of recovery and growth.

To date, the most successful techniques for storing *B. napus* germplasm do not employ cryoprotective agents, other than those agents found in the regular culture medium. The most effective *B. napus* germplasm storage techniques include the use of air-dried microspore-derived embryos (Uragami et al. 1993) and/or slow cooling of microspores followed by cryostorage (Charne et al. 1988; Chen and Beversdorf 1992a,b). Cryopreservation of air-dried microspore-derived embryos encapsulated in calcium-alginate gel produced survival rates which exceeded 90% (Uragami et al. 1993). However, since it is difficult to remove the gel, which hinders regeneration of embryos into plants, the efficiency of this technique requires further improvement. Cryopreservation of microspores has not produced the same survival rates, relative to controls, as has cryopreserved air-dried or gel-encapsulated embryos. However, cryopreservation of

microspores requires far less manipulation and a larger amount of germplasm can be stored in a much smaller amount of space resulting in increased efficiency in a breeding program.

To improve survival frequencies of cryo-preserved microspores, cold acclimation may be of use. Cold acclimated plant cells are better able to withstand the effects of low temperature and freezing. Cold acclimation causes plant cells to undergo a number of changes which allow for physiological adaptation to reduced temperature (Guy 1990). *B. napus* cells, embryos and whole plants can be stimulated to undergo various changes associated with cold acclimation. These changes can lead to increased ability to withstand the damaging effects of a freeze-thaw cycle. Alterations in the fatty acid composition of the plasma membrane, protein synthesis and related changes are known to occur (Diepenbrock 1984; Meza-Basso et al. 1986; Johnson-Flanagan and Singh 1987; Parra et al. 1990; Williams et al. 1992; Saez-Vasquez et al. 1993). Treating *B. napus* cells, embryos and seeds with dessication stress or abscicic acid (ABA) also induces similar alterations (Harada et al. 1989; Orr et al. (1986, 1990); Johnson-Flanagan et al. (1991, 1992); Weretilnyk et al. 1993).

Previous experiments in cryopreservation of microspores utilized microspores derived from donor plants grown at 23/19^{0}C (day/night) (Charne et al. 1988; Chen and Beversdorf 1992a,b). These temperatures would therefore not induce any cold-acclimating changes. In the present experiment there were two initial phases during which microspores may have undergone changes which resulted in better survival at low temperature. Microspore donor plants were initially grown at low temperature, 10/5^{0}C (day/night), and

the isolated buds were subsequently placed on ice for 24 hours before microspore isolation and treatment. Growing donor plants at reduced temperature produces a population of microspores which is initially more embryogenic and which may be better acclimated to withstand freezing and cryopreservation.

In addition to the pre-conditioning effects of lowered temperature, survival during cryopreservation may further be improved by use of cryoprotectants. In previous research work on cryopreservation of *B. napus* microspores, the primary cryoprotectant medium used was standard NLN culture medium supplemented with 13% sucrose (Charne et al. 1988, Chen and Beversdorf 1992a,b). Sucrose occurs as a natural, relatively non-toxic cryoprotectant in plant cells and its levels usually increase in cells which are subjected to cold hardening conditions (Koster and Lynch 1992). Sucrose can stabilize proteins and membranes and prevent their interaction with toxic constituents of a concentrated cytoplasm, as occurs with cooling to subzero temperatures (Anchordoguy et al. 1987; Lee and Timasheff 1981; Strauss and Hauser 1986). As well, increased levels of sucrose in the cryoprotectant medium can serve to decrease the water potential of the cell cytoplasm which further depresses the freezing point of cytoplasmic water. This can serve to reduce damage from ice formation.

In this experiment, samples placed in NLN containing 13% sucrose and cooled to -15^{0}C at -0.5^{0}C/min. produced the highest embryogenic frequencies of 71.8 and 77.3% that of the control for non-immersed and liquid nitrogen immersed samples respectively (Figure 3.4.1). In the other treatments in which samples were placed in liquid nitrogen, embryogenic frequencies were comparable to non-immersed counterparts. Although the

25% sucrose samples appeared to have higher survival frequencies at lower terminal temperatures, survival tended to decrease as sucrose concentration increased and as terminal cooling temperature decreased (Figure 3.4.1, 3.4.4, 3.4.7). The lowest embryogenic frequencies, 30.0 and 30.7%, were obtained for microspores placed in 50% sucrose that were cooled to -60^{0}C with and without liquid nitrogen immersion respectively (Figure 3.4.7).

In a previous experiment, 3.3 of this thesis, microspores from buds that did not receive ice pre-treatment were placed in 13% sucrose and subjected to the same cooling regime. Liquid nitrogen immersion was lethal at terminal temperatures of -15 and -30^{0}C. Only when cooled to terminal temperatures of -45 and -60^{0}C, did the samples survive liquid nitrogen immersion with embryogenic frequencies of approximately 1/3 that of the control. Since dehydration of the cytoplasm increases in a temperature dependent manner, as the terminal temperature decreases, it is believed that a sufficient amount of cytoplasmic dehydration did not occur until the lower ranges of terminal temperatures were reached.

Initial experimental work by Charne et al. (1988) showed that cryopreservation of *B. napus* cv. Topas microspores resulted in decreased frequency of embryogenesis after treatment. Nitrogen-treated microspores produced an average embryogenic frequency of only 8.0% that of the untreated control. A cryoprotectant medium consisting of NLN culture medium supplemented with 13% sucrose, cooling rate of 0.25^{0}C/min., and terminal temperature of -15^{0}C before immersion in liquid nitrogen were used. Chen and Beversdorf (1992b) used the same cryoprotectant medium and cooling rate but found that a terminal

temperature of -35°C' produced optimal results. With *B. napus* genotypes G-231 and M3-124, nitrogen immersed material produced embryogenic frequencies of 18 and 40% that of the control respectively.

The experiments of Charne et al. (1988) and Chen and Beversdorf (1992b) did not involve use of donor plants grown at reduced temperatures or 24 hour ice pre-treatment of donor buds which could have affected microspore survival and subsequent embryogenesis. The results suggest that the ice pre-treatments used in this experiment induced acclimatizing changes which had a major influence on preventing mortality from cryo-damage following liquid nitrogen storage. Microspores conditioned in this manner may have the ability to become dehydrated more readily and are therefore less susceptable to ice damage during and after liquid nitrogen immersion. However, this may also cause microspores to be more susceptable to dehydration damage if placed in a cryoprotectant solution having high osmotic concentration or if allowed to cool to excessively low terminal temperatures. In this experiment embryogenesis tended to decrease as sucrose concentration increased and as the terminal temperature decreased. Possibly this is suggestive of increased damage due to excessive dehydration.

Cryopreservation of plant cells is generally considered to be an ideal way to store germplasm in a genetically stable form (Withers 1984). Chromosome doubling is an occurrence not normally associated with cryopreservation of plant cells. Differences between cell types, stage of development and stage of the cell cycle may exist which protect or predispose cells to chromosome doubling. Cryo-induced chromosome doubling in *B. napus* has, to date, only been shown to occur with late uninucleate stage

microspores (Charne et al. 1988; Chen and Beversdorf 1992a). However, it has been known for several years that temperature shocks can cause alterations during cell divison in pollen and microspores which lead to chromosome doubling (Sax 1936, 1937).

The mechanism by which cryopreservation induces chromosome doubling at the microspore stage of development is not yet known. Some researchers have suggested that chromosome doubling in *B. napus* microspores may result from endoreplication or endomitosis (Keller et al. 1975). Others have suggested that cryo-selection of pre-existing unreduced microspores may be involved (Chen and Beversdorf 1992a). However, it is unlikely that larger cells, such as unreduced gametes, would have a selective advantage. The larger volume of these cells prevents removal of water which should lead to more ice-damage and increased mortality. Most likely, sub-lethal cryo-damage to components of the cytoskeleton, such as tubulin protein and membrane systems, is involved.

Cryo-induced chromosome doubling frequency in *B. napus* microspores has been shown to increase as the terminal cooling temperature decreases during the cryopreservation protocol (Chen and Beversdorf 1992a). Dehydration of the cytoplasm during cooling, which is dependent on terminal cooling temperature, can cause a number of changes which affect cell division. Precipitation of proteins, alterations in the plasma membrane and changes in cytoplasmic calcium concentration can occur as a result of dehydration (Dowgert and Steponkus 1984; Lynch and Steponkus 1989; Okazaki and Tazawa 1990; Tamiya et al. 1985). In turn, these changes may cause damage to microtubule networks involved with the first cell division of microspores in culture (Hause et al. 1992). Consequently, an increased proportion of microspores may bypass

one round of cell division. Since late uninucleate microspores, which are in G2 and S phases of the cell cycle, have already doubled chromosome numbers, a cell which is diploidized subsequently is the result. Such an event can be considered to be equivalent to endomitosis; as observed with colchicine treatment of plant cells (Blackmore and Tootill 1984; Brodsky and Uryvaeva 1985). Further research is required to better characterize the events which occur during cryo-induced chromosome doubling of *B. napus* microspores.

In the present experiment, the control diploidization frequency was 29.3%. With all of the treatments, cooled to -15^{0}C and lower, diploidization frequencies of 63.7% or higher were obtained (Figure 3.4.3, 3.4.6, 3.4.9). There did not appear to be any relationship between terminal temperature, sucrose concentration or liquid nitrogen immersion and frequency of diploidization. In the critical period between 0 and -15^{0}C some significant event may be occurring which increases the frequency of diploidization in the ice pre-treated microspores. Since embryogenic and diploidization frequencies were generally high regardless of liquid nitrogen immersion, it is possible that a significant amount of dehydration may have occurred before the microspore samples reached -15^{0}C. If the level of dehydration is related to both survival (embryogenesis) and diploidization, this may explain the results.

In a previous experiment, 3.3 of this thesis, microspores from buds that did not receive ice pre-treatment were placed in 13% sucrose and subjected to the same cooling regime. Doubling frequencies increased as terminal temperatures decreased. Liquid nitrogen immersion did not appear to have a significant effect on diploidization frequency.

Maximal diploidization values of 87.5 and 79.9% were obtained at a terminal temperature of -60^{0}C for non-immersed and liquid nitrogen immersed samples respectively. The control in experiment 3.3 produced a diploidization frequency of 22.5%.

Charne et al. (1988) showed that cryopreservation of *B. napus* cv. Topas microspores resulted in reduced embryogenesis after treatment. However, the frequency of diploidization in plants derived from cryo-treated microspores, cooled to -15^{0}C and stored in liquid nitrogen, was 88.0% compared to the control value of 7.0%. Chen and Beversdorf (1992b) demonstrated that the frequency of diploidization increased as the terminal cooling temperature decreased. Immersion in liquid nitrogen was shown to have no effect on diploidization frequency. With the *B. napus* genotypes G-231 and M3-124, at a terminal temperature of -40^{0}C, they obtained maximal diploidization frequencies of 51.1 and 47.7% compared to control values of 22.3 and 20.4% respectively.

Combined, the effects of growing donor plants at decreased temperatures, ice pre-treatment of buds, and cryo-treatment procedure increased survival and chromosome doubling of *B. napus* microspores. These treatments may lead to improved germplasm storage techniques, development of microspore derived lines, and better utilization of germplasm resources. Only one cultivar was tested in this experiment, therefore, further research is required to determine if the same treatments are useful for other *B. napus* germplasm.

Table 3.4.1 Mean percentage values for embryo yield, plant regeneration and diploidization following ice (0°C) and cryo-treatment of *B. napus* microspores.

Sucrose (% w/v)	Terminal Temp.	N_i (+/-) (-196 °C)	% Embryo Yield	% Plant Regeneration	% Diploids
13	0 °C	-	100.0 a	38.8 abc	29.3 c
13	-15 °C	-	71.8 bcd	31.3 abcd	70.1 ab
		+	77.3 b	42.5 abc	68.6 ab
	-30 °C	-	64.2 cdef	25.0 bcd	79.2 ab
		+	76.9 bc	41.3 abc	73.3 ab
	-45 °C	-	34.6 kl	30.0 abcd	67.9 ab
		+	36.7 kl	21.3 cd	74.2 ab
	-60 °C	-	35.6 kl	27.5 abcd	88.3 ab
		+	31.3 l	31.3 abcd	83.5 ab
25	-15 °C	-	68.4 bcdef	33.8 abcd	67.3 ab
		+	44.5 ijk	33.8 abcd	78.3 ab
	-30 °C	-	58.2 efgh	50.0 a	72.3 ab
		+	70.5 bcde	48.8 ab	75.3 ab
	-45 °C	-	61.9 defg	23.8 cd	63.7 b
		+	61.4 defg	28.8 abcd	64.2 ab
	-60 °C	-	55.8 fghi	31.3 abcd	78.1 ab
		+	50.0 ghij	36.3 abcd	93.7 a
50	-15 °C	-	42.0 jkl	21.3 cd	71.8 ab
		+	40.4 jkl	37.5 abc	78.9 ab
	-30 °C	-	34.7 kl	21.3 cd	83.3 ab
		+	35.3 kl	12.5 d	86.7 ab
	-45 °C	-	46.5 hijk	28.8 abcd	75.0 ab
		+	45.4 ijk	25.0 bcd	91.4 ab
	-60 °C	-	30.0 l	31.3 abcd	70.8 ab
		+	30.7 l	20.0 cd	74.2 ab

Note: Embryo percentages are calculated as a percentage of the control in which 2790.0 (± 105.6) embryos per 6 x 10^5 plated microspores were produced. Results are based on four replicates with 20 embryos transferred per replicate.

Values which share the same letter(s) are not significantly different from each other (@ P= 0.05) according to Duncan's multiple range test.

The pooled standard error values were ±125.8 embryos per 6 x 10^5 plated microspores or ±4.5% (embryo yield), ±7.1% (plant regeneration) and ±8.5% (diploidization).

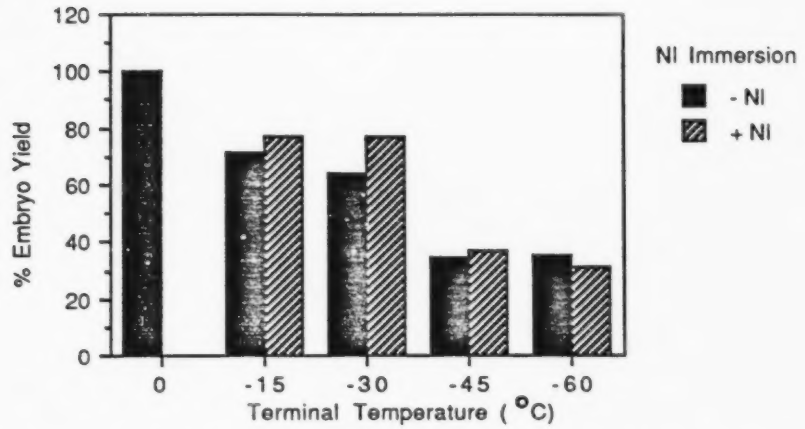

Figure 3.4.1 Embryo yield from *B. napus* microspores stored in 13% sucrose and treated with different terminal temperatures and (±) liquid nitrogen immersion. (Pooled SE= ±4.5%).

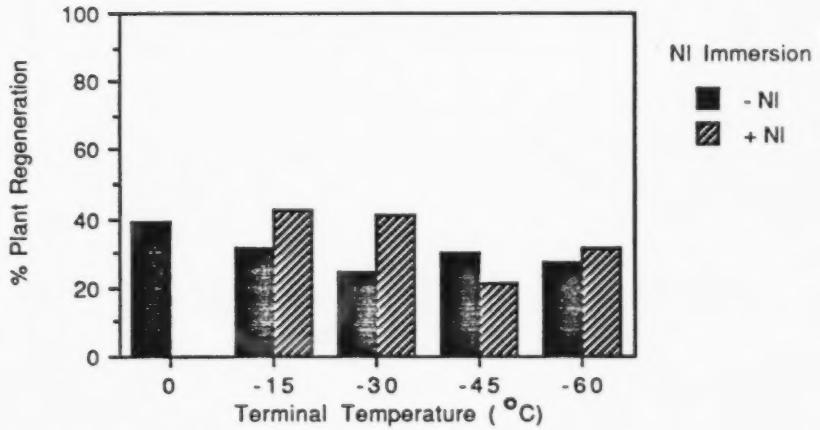

Figure 3.4.2 Plant regeneration from *B. napus* microspores stored in 13% sucrose and treated with different terminal temperatures and (±) liquid nitrogen immersion. (Pooled SE= ±7.1%).

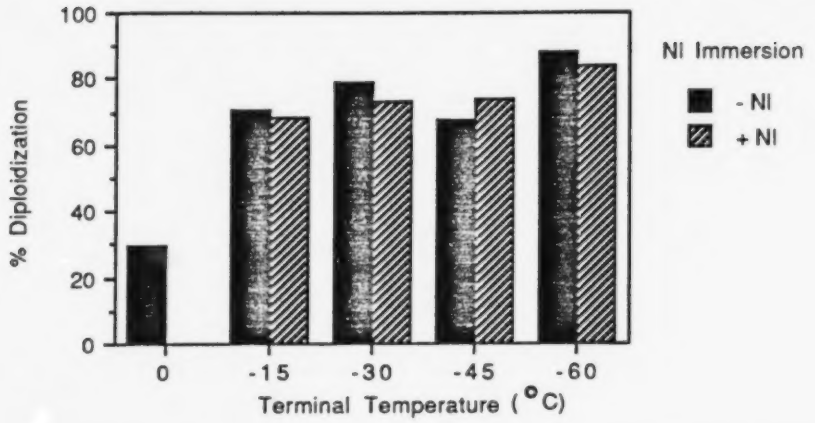

Figure 3.4.3 Diploidization frequencies from *B. napus* microspores stored in 13% sucrose and treated with different terminal temperatures and (±) liquid nitrogen immersion. (Pooled SE= ±8.5%).

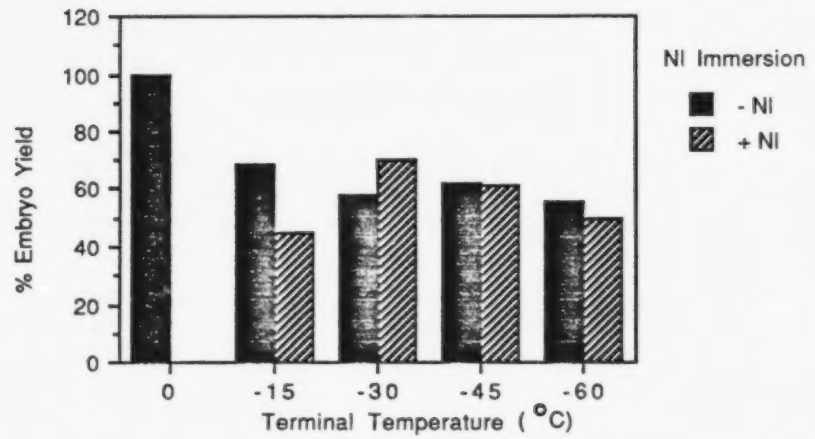

Figure 3.4.4 Embryo yield from *B. napus* microspores stored in 25% sucrose and treated with different terminal temperatures and (±) liquid nitrogen immersion. (Pooled SE= ±4.5%).

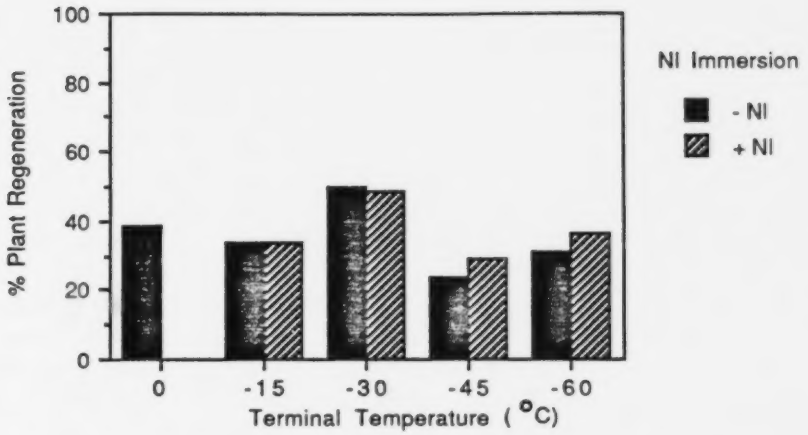

Figure 3.4.5 Plant regeneration from *B. napus* microspores stored in 25% sucrose and treated with different terminal temperatures and (±) liquid nitrogen immersion. (Pooled SE= ±7.1%).

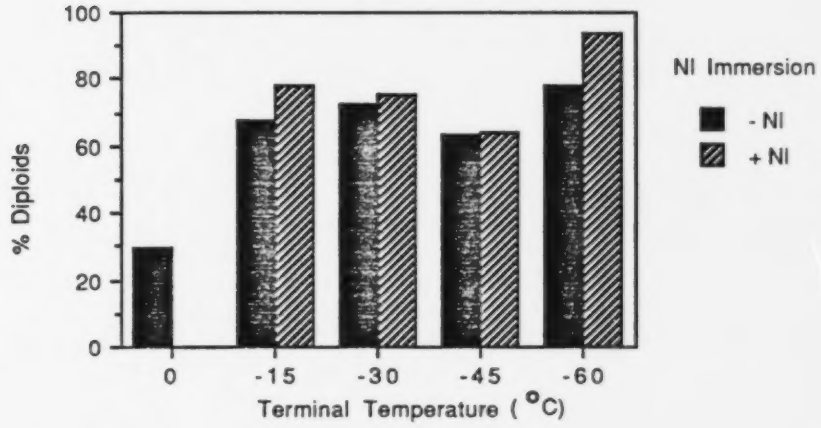

Figure 3.4.6 Diploidization frequencies from *B. napus* microspores stored in 25% sucrose and treated with different terminal temperatures and (±) liquid nitrogen immersion. (Pooled SE= ±8.5%).

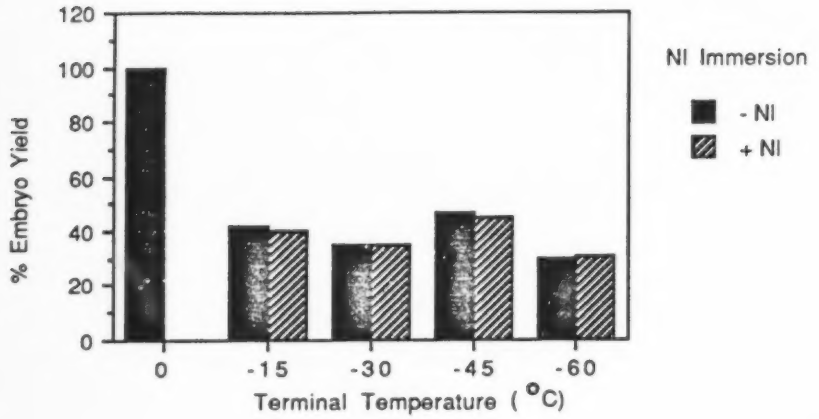

Figure 3.4.7 Embryo yield from *B. napus* microspores stored in 50% sucrose and treated with different terminal temperatures and (±) liquid nitrogen immersion. (Pooled SE= ±4.5%).

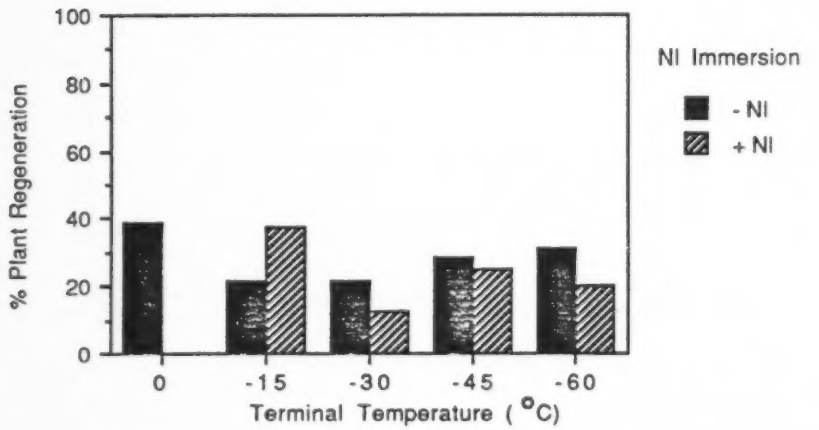

Figure 3.4.8 Plant regeneration from *B. napus* microspores stored in 50% sucrose and treated with different terminal temperatures and (±) liquid nitrogen immersion. (Pooled SE= ±7.1%).

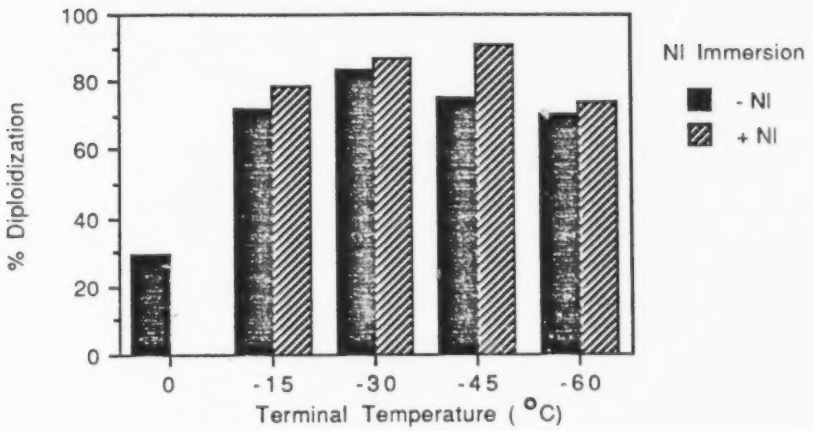

Figure 3.4.9 Diploidization frequencies from *B. napus* microspores stored in 50% sucrose and treated with different terminal temperatures and (±) liquid nitrogen immersion. (Pooled SE= ±8.5%).

3.4.6 Literature Cited

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4.0 SUMMARY AND CONCLUSIONS

The ability of five anti-microtubule agents and cryo-treatments to induce chromosome doubling in microspore-derived *Brassica napus* L. cv. Topas were evaluated. Amiprophos-methyl, oryzalin, pronamide, trifluralin and colchicine were applied to microspores and embryos (30 day old) at various concentrations and periods of duration. Freshly isolated and ice pre-treated (0^0C) microspores were subjected to cryo-treatments consisting of cooling at -0.5^0C per minute to terminal temperatures of 0, -15, -30, -45, and -60^0C with and without subsequent immersion in liquid nitrogen (-196^0C).

Microspores treated with pronamide (15 μM) and trifluralin (1.0 μM) for 24 hours had chromosome doubling frequencies of 82.6 and 83.9% respectively, and improved microspore embryogenesis. Colchicine (1.25 mM) treatment resulted in a doubling frequency of 80.4% and, relative to the control, slightly reduced embryogenesis. Although amiprophos-methyl and oryzalin increased chromosome doubling, the concentrations effective to do so resulted in decreased embryogenesis.

Treatment of embryos (30 day old) with oryzalin and trifluralin (10 μM for four days) resulted in doubling frequencies of 78.6 and 61.1% respectively. Amiprophos-methyl, pronamide and colchicine treatment of embryos resulted in doubling frequencies of less than 50%. Due to the requirement for more procedural steps and lower chromosome doubling frequencies, it was concluded that treatment of embryos with anti-microtubule agents was less effective than treatment of microspores for inducing chromosome doubling.

Freshly isolated microspores placed in 13% sucrose and cooled at -0.5^0C per

minute to different terminal temperatures illustrated the effect terminal cooling temperature had on chromosome doubling. As the terminal temperature decreased, the percentage of diploid microspore-derived plants produced from treated microspores increased. Samples cooled to -15, -30, -45 and -60^{0}C produced doubling frequencies of 39.5, 44.4, 63.8 and 87.5% respectively. Immersion of microspores in liquid nitrogen was lethal unless samples were cooled to at least -45 and -60^{0}C before immersion resulting in embryogenic (survival) frequencies of 37.0 and 31.7% that of the control respectively. Although survival was reduced by liquid nitrogen immersion, embryos produced from these samples had increased plant regeneration frequencies. Due to the lower population density and reduced competition for nutrients embryos from these samples may have been in better condition to germinate and develop into plants. Liquid nitrogen immersion did not significantly alter the doubling frequency relative to the non-immersed samples.

Pre-treatment of microspore donor buds for 24 hours with ice (0^{0}C) before cryo-treatment significantly improved survival of microspores following liquid nitrogen immersion. At a terminal temperature of -15^{0}C non-immersed and immersed samples, placed in 13% sucrose, produced embryogenic (survival) frequencies of 71.8 and 77.3% that of the control value respectively. The percentage of diploids produced by these treatments were 70.1 and 68.6% respectively. Cooling of ice pre-treated samples to terminal temperatures lower than -15^{0}C or use of cryoprotectant media with increased sucrose concentration caused embryogenesis (survival) frequencies to decline. This may suggest that excessive removal of intracellular water and/or damage from increased solute concentration had occurred.

Overall, application of pronamide (15.0 μM) and trifluralin (1.0 μM) to freshly isolated microspores for 24 hours is the most efficient means of doubling the chromosome number in microspore-derived *B. napus* L. cv. Topas. These treatments are easily incorporated into the regular microspore culture protocol, require extremely low initial cost inputs, little additional time, and result in relatively high (> 80%) chromosome doubling frequencies. As well, these treatments can increase microspore embryogenesis, result in complete chromosome doubling, produce plants which have high seed set and eliminate the need for an extra generation for seed increase. Although cryo-treatment of microspores results in increased chromosome doubling (70 to 80%), it is more expensive and requires more procedural steps than do treatments involving anti-microtubule agents. However, in combination with ice pre-treatment, storage of microspores in liquid nitrogen is an effective method for germplasm storage which has the added benefit of increasing the chromosome doubling frequency.

5.0 Appendices

Appendix A:

B5 Wash Medium (1L)

- B5x 10 stock (below)	100 mL
- sucrose	130 g
- ddH_2O added to a final volume of 1 L.	
- stir contents until dissolved.	
- pH adjusted to 6.0.	
- sterilize in autoclave.	

B5x 10 (frozen stock) (1 L)

- KNO_3	12.50 g
- $MgSO_4$-$7H_2O$	1.25 g
- $CaCl_2$-$2H_2O$	3.75 g
- $(NH_4)_2SO_4$	0.67 g
- NaH_2PO_4-H_2O	0.75 g
- Fe 330	0.20 g
- B5x 10 vitamin stock (below)	50.00 mL
- B5x 100 micronutrient stock (below)	50.00 mL
- KI stock (below)	5.00 mL
- ddH_2O added to a final volume of 1 L.	
- stir contents until dissolved.	
- store in freezer as 100 mL samples.	

B5x 10 (frozen vitamin stock) (1 L)

- myoinositol	10.00 g
- nicotinic acid	0.10 g
- pyridoxine HCl	0.10 g
- thiamine HCl	1.00 g
- ddH_2O added to a final volume of 1 L.	
- stir contents until dissolved.	
- store in freezer as 100 mL samples.	

B5x 100 (frozen micronutrient stock)

- $MnSO_4$-H_2O	1.00 g
- H_3BO_3	0.30 g
- $ZnSO_4$-$7H_2O$	0.20 g
- Na_2MnO_4-$2H_2O$	0.025 g
- $CuSO_4$-$5H_2O$	0.0025 g
- $CoCl_2$-$6H_2O$	0.0025 g

- ddH_2O added to a final volume of 1 L.
- stir contents until dissolved.
- store in freezer as 100 mL samples.

KI Stock

- KI	0.83 g

- ddH_2O added to a final volume of 1 L.
- stir until contents dissolved.
- refrigerate (2-5^{0}C).

Appendix B:

NLN Culture Medium (1 L)

- ddH_2O	500 mL
- KNO_3	0.125 g
- $MgSO_4$-$7H_2O$	0.125 g
- $Ca(NO_3)_2$-$4H_2O$	0.50 g
- KH_2PO_4	0.125 g
- Fe 330	0.04 g
- B5x 100 vitamin stock (Appendix A)	10.00 mL
- B5x 100 micronutrient stock (Appendix A)	10.00 mL
- glutathione	0.03 g
- L-glutamine	0.80 g
- L-serine	0.10 g
- sucrose	130 g

- ddH_20 added to a final volume is 1 L.
- stir contents until dissolved.
- pH adjusted to 6.0.
- filter sterilize and store in fridge (2-5^{0}C).

Appendix C:

B5 Solid Culture Media (1L)

- B5x 10 stock (Appendix A)	200 mL
- sucrose	20 g
- GA_3 (0.15 mg/l)	1 mL
- ddH_20 added to a final volume of 1 litre.	
- stir contents until dissolved.	
- pH adjusted to 5.7.	
- agar	8 g
- sterilize in autoclave.	
- pour 10 ml into petri plates (100x 15mm).	
- approximately 100 plates can be poured.	
- allow samples to solidify.	
- seal in plastic bags until used.	